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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Papel das Purinas Endógenas (ATP e Adenosina) em Pacientes com Disfunção do Aparelho Urinário Inferior

Role of Endogenous Purines (ATP and Adenosine) in Patients with Lower Urinary Tract Dysfunction.

MIGUEL ANTÓNIO COSTA DE ARAÚJO DA SILVA RAMOS

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Orientador:
Nome: Doutor Paulo Correia-de-Sá
Categoria: Professor Catedrático
Afiliação: Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

Coorientador:
Nome: Doutor Lafuente de Carvalho
Categoria: Professor Catedrático Convidado
Afiliação: Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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ABBREVIATIONS

2-Cl-IBMECA, 2-chloro-N^6-(3-iodobenzyl) adenosine-5'-N-methylcarboxamide

5'NTase, 5'-nucleotidase

α,β-meATP, α,β-Methyleneadenosine 5′-triphosphate

ABC proteins, ATP-binding cassette transporters

ACh, acetylcholine

ADA, adenosine deaminase

ADO, adenosine

ADP, adenosine diphosphate

ADPβS, adenosine 5′-[β-thio]diphosphate

AMP, adenosine monophosphate

ANOVA, analysis of variance

AP, alkaline phosphatases

ARL67156, 6-N,N-diethyl-d-β,γ-dibromomethylene ATP trisodium salt

ATP, adenosine triphosphate

AUC, area under the curve

BDNF, brain-derived neurotrophic factor

BoNT-A, botulinum neurotoxin A

BOO, bladder outlet obstruction

BPH, benign prostatic hyperplasia

cAMP, cyclic adenosine monophosphate

CCPA, 2-choloro-N^6-cylcopentyladenosine

CHA, N^6-cyclohexyladenosine

ChAT, choline acetyltransferase

CHP, Centro Hospitalar do Porto

CNS, central nervous system

Cr, creatinine

DAG, diacylglycerol

DMSO, dimethyl sulfoxide

DMPX, 3,7-dimethyl-1-propargyl-xanthine

DPCPX, 1,3-dipropyl-8-cyclopentylxanthine

DU, detrusor underactivity

EFS, electrical field stimulation

EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine

ELISA, enzyme-linked immunoabsorbent assay

ENaC, amiloride-sensitive epithelial Na^+ channels

E-NPP, ecto-nucleotide pyrophosphatases

E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase

HPLC, high performance liquid chromatography

HX, hypoxanthine

ICBAS, Instituto de Ciencias Biomédicas Abel Salazar

ICS, International Continence Society

INO, inosine

IP3, inositol 1,4,5-trisphosphate

IPSS, International Prostate Symptom Score

LUTD, lower urinary tract dysfunctions

LUTS, lower urinary tract symptoms

LDH, lactate dehydrogenase

NAD, nicotinamide adenine dinucleotide

NADH, nicotinamide adenine dinucleotide plus hydrogen

NANC, non-adrenergic non-cholinergic

NECA, 5'-*N*-ethylcarboxamidoadenosine

NGF, nerve grow factor

NO, nitric oxide

OAB, overactive bladder

PBS, phosphate saline buffer

PDGFR-α, a platelet derived growth factor receptor α

PLC, phospholipase C

PSA, prostatic specific antigen

PPADS, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid

PVR, postvoid residual volume

Qmax, maximal flow rate

QOL, quality of life

ROC, receiver operating characteristic

R-PIA, N^6-(R-2-phenylisopropyl)adenosine

RT-PCR, reverse transcription polymerase chain reaction

SD, standard deviation

SK, small conductance Ca^{2+}-activated K^+ channels

SNAP25, synaptosome-associated protein of 25 kDa

SNARE, N-ethylmaleimide–sensitive factor attachment receptor

TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate

TRPV1, transient receptor potential cation channel subfamily V member 1

TTX, tetrodotoxin

UDP, uridine diphosphate

UTP, uridine triphosphate

VAChT, ACh vesicular transporter

VV, voided volume

ABSTRACT

Lower urinary tract symptoms (LUTS) are very prevalent worldwide, both in men and women. They cause a significantly negative impact on quality of life in both patients and their relatives. Due to multifactorial origin, the diagnosis of the underlying condition is difficult to obtain and treatment options are insufficient. Purinergic signalling pathways are now considered to play an important role in regulating urinary bladder function. However, most of the data concerning purinergic signalling in the lower urinary tract comes from studies in animal models and little is known about this pathway in humans, particularly in those with lower urinary tract dysfunctions (LUTD). The present work was designed to fill the gaps in our knowledge regarding the role of adenosine triphosphate (ATP) and adenosine (ADO) on afferent and efferent paths of the micturition reflex. Results indicate that the detrusor of patients with benign prostatic hyperplasia (BPH) obtained during open prostatectomy release significantly more ATP and acetylcholine (ACh) when stimulated electrically than control samples collected from organ donors. The increase in the purinergic tone is favoured by an impairment in ATP hydrolysis and, subsequent, ADO formation due to deficient activity of ecto-nucleoside triphosphate diphosphohydrolase-1 (E-NTPDase-1/CD39) and ecto-5'nucleotidase (5'NTase/CD73) in these patients. The higher ATP bioavailability and deficient ADO formation end-up by favouring ACh release, because ATP facilitates whereas ADO inhibits ACh release through the activation of prejunctional P2X3 and A_1 receptors, respectively. Comparing the rate of ATP hydrolysis in the luminal with the abluminal sides of the human urothelium, we concluded that it was faster in the suburothelium, which may in part be due to surplus activity of the E-NTPDase2 that is a preferential triphosphatase resulting in significant ADP accumulation. Excessive urothelial ATP release and accumulation in the lumen of the bladder may concur to increase the urinary concentrations of the nucleotide in patients with lower urinary tract dysfunctions (LUTD), which might be of clinical relevance. As a matter of fact, we show here that female patients with overactive bladder (OAB), as well as men with bladder outlet obstruction (BOO) due to BPH, have significantly higher urinary ATP than healthy controls. Urinary ATP concentrations correlated with the voided volume, suggesting that bladder distension promotes the release of

ATP from the urothelium, which is in keeping with *in vitro* experiments. We also show here for the first time that urinary ATP correlates inversely with flow rate, suggesting that ATP release may be related to the degree of obstruction. This raises the possibility that urinary ATP amounts might be the first non-invasive pressure-transducer biomarker in the diagnosis of LUTD. Usefulness of urinary ATP as a dynamic biomarker in LUTD was further strengthen since its levels decreased significantly after intravesical botulinium toxin-A injections used to improve OAB symptoms.

In conclusion, the results presented in this thesis add compelling information to understand the pathophysiological mechanisms responsible for the increase in the bladder purinergic tone verified both in the mucosa and detrusor of patients with LUTD and foresee the clinical application of urinary ATP as a dynamic biomarker of LUTD. Moreover, we anticipate that drugs targeting purinergic signalling pathways placed in evidence in the present study, may be useful novel candidates for therapeutic intervention in LUTS.

RESUMO

Os sintomas do aparelho urinário baixo são muito prevalentes em homens e mulheres de todo o mundo e têm um impacto negativo muito significativo na qualidade de vida dos pacientes e das suas famílias. A abordagem clínica destes sintomas é difícil pela sua natureza multifatorial e as soluções terapêuticas disponíveis atualmente são insuficientes. O sistema purinérgico tem reconhecidamente um papel regulador em vários mecanismos envolvidos no funcionamento da bexiga. No entanto, a maior parte do conhecimento adquirido sobre este assunto resulta de estudos em modelos animais, sendo escassos os estudos em humanos, particularmente em doentes com disfunções do aparelho urinário inferior. Este trabalho foi delineado para preencher algumas lacunas no conhecimento do papel sinalizador do ATP e da adenosina no componente aferente e eferente do reflexo miccional. Os resultados mostram que a estimulação elétrica do detrusor de doentes com hiperplasia benigna da próstata (HBP) isolado durante prostatectomia aberta liberta mais ATP e acetilcolina (ACh) do que o detrusor de indivíduos controlo (dadores cadavéricos de órgãos). O aumento do tónus purinérgico é potenciado pela redução da velocidade de hidrólise do ATP e, subsequente, redução da formação de adenosina devidos à menor atividade da E-NTPDase-1/CD39 e da ecto-5´NTase/CD73 nos pacientes. O desequilíbrio entre a metabolização do ATP e a formação de adenosina em favor da acumulação do primeiro favorece a libertação de ACh através da ativação de recetores pré-sinápticos excitatórios do tipo P2X3 e da perda do tónus inibitório mediado pelos recetores A_1 da adenosina. Comparando a cinética do catabolismo extracelular do ATP do lado luminal e abluminal do urotélio verificámos que a mesma era mais rápida na região suburotelial levando a acumulação de ADP mercê da presença da E-NTPDase2, que é uma trifosfatase responsável pela metabolização preferencial de ATP com menor afinidade para o ADP. A libertação de ATP pelo urotélio e a sua acumulação excessiva no lúmen da bexiga favorece o aumento dos níveis urinários deste nucleótido em doentes com disfunções do trato urinário inferior, fato que pode ter relevância clínica. Com efeito, os resultados mostram que as concentrações urinárias de ATP são mais elevadas em mulheres com bexiga hiperativa, assim como em homens com obstrução infravesical por HBP, do que as verificadas em

controlos saudáveis. Os níveis de ATP urinário correlacionam-se com o volume urinado confirmando resultados "in vitro" onde se demonstrou que a distensão da bexiga promove a libertação do nucleótido pelo urotélio. Também verificámos neste trabalho pela primeira vez a existência de uma correlação positiva entre os níveis de ATP urinário e (1) a gravidade dos sintomas urinários em mulheres com bexiga hiperativa e (2) com a severidade da obstrução infravesical nos homens com HBP avaliada em condições isovolumétricas. Estes últimos resultados mostram que o ATP urinário pode ser considerado o primeiro biomarcador não-invasivo de pressão intravesical com utilidade nas disfunções do aparelho urinário inferior. A utilidade clínica do doseamento dos níveis de ATP urinário como biomarcador dinâmico nas disfunções do aparelho urinário inferior foi comprovada já que os seus níveis baixaram significativamente após injeções intravesicais de toxina botulinica-A destinadas à melhoria sintomática da hiperatividade vesical.

Em conclusão, os resultados apresentados nesta tese ajudam a compreender melhor os mecanismos responsáveis pelo aumento do tónus purinérgico na bexiga (mucosa e detrusor) de doentes com disfunções do trato urinário inferior e antevê possíveis aplicações clínicas do ATP urinário como biomarcador não-invasivo nas disfunções miccionais. Mais ainda, os resultados demonstram que a manipulação da sinalização purinérgica em várias vertentes, locais de libertação, enzimas e recetores, pode constituir uma nova estratégia para o tratamento dos sintomas do aparelho urinário inferior.

SCIENTIFIC OUTPUTS

Papers published in peer-reviewed international journals

2013 "**Urinary ATP may be a dynamic biomarker of detrusor overactivity in women with overactive bladder syndrome"**
Silva-Ramos, M., Silva, I., Oliveira, O., Ferreira, S., Reis, M. J., Oliveira, J. C., & Correia-de-Sá, P.
PLoS One, 8(5), e64696. doi:10.1371/journal.pone.0064696.

2015 "**Impairment of ATP hydrolysis decreases adenosine A1 receptor tonus favoring cholinergic nerve hyperactivity in the obstructed human urinary bladder"**
Silva-Ramos, M., Silva, I., Faria, M., Magalhães-Cardoso, M. T., Correia, J., Ferreirinha, F., & Correia-de-Sá, P.
Purinergic Signal, 11(4), 595-606. doi:10.1007/s11302-015-9478-z.

2016 "**Increased urinary adenosine triphosphate in patients with bladder outlet obstruction due to benign prostate hyperplasia"**
Silva-Ramos, M., Silva, I., Oliveira, J.C. & Correia-de-Sá, P.
Prostate, 2016 Jul 15. doi: 10.1002/pros.23207.

"ATP promotes the release of acetilcholine from stimulated detrusor strips of BPH patients viaP2X2/3 receptors activation"
Silva-Ramos, M., Silva, I., Faria, M., Magalhães-Cardoso, M.T., Correia, J., Ferreirinha, F. & Correia-de-Sá, P.
in preparation

"Intravesical botulinum neurotoxin-a injections decreases urinary ATP concentration in patients with overactive bladder syndrome"
Silva-**Ramos, M., Silva, I., Reis, D., Oliveira, J.C. & Correia-de-Sá, P.**
in preparation

Book Chapters

2015 "**Urinary bladder disorders: Is adenosine friend or foe?"**

Silva-Ramos, M., Silva, I., Faria, M., Magalhães-Cardoso, M. T., & Correia-de-Sá, P

In K. Warrick (Ed.), *Adenosine receptors: Pharmacology, functions and therapeutic aspects* (1st ed., pp. 115-142). Hauppauge, NY: Nova Science Publishers Inc.. ISBN: 978-1-63463-454-0.

2016 "**Overactive bladder (OAB): Is there a place for drugs targeting the purinergic cascade?"**

Silva-Ramos, M., Silva, I., Ferreirinha, F., Faria, M. & Correia-de-Sá, P.

In E. Larson (Ed.), *Overactive Bladder (OAB): Prevalence, Risk Factors and Management* (1st ed.). Hauppauge, NY: Nova Science Publishers Inc. ISBN: 978-1-63485-033-9.

Patent

2016 "**ATP as a biomarker of bladder outlet obstruction"**

CHAPTER 1

INTRODUCTION

“The prostate should be exonerated from always being the first suspect for crimes against the micturition”

Vicenzo Mirone

1.1 LOWER URINARY TRACT SYMPTOMS

The main function of the lower urinary tract is the storage and periodic elimination of urine. It requires the coordinated activity of two functional units: a reservoir (the urinary bladder) and an outlet consisting of the bladder neck, the urethra, and the urethral sphincter. Coordination between these organs is mediated by a complex neural control system located in the brain, spinal cord, and peripheral ganglia. Thus, urine storage and release are highly dependent on central nervous system pathways in addition to local circuitry. This distinguishes the lower urinary tract from other visceral systems (e.g., the gastrointestinal tract and cardiovascular). Another particular feature of the lower urinary tract is that we consciously control de autonomic nervous responses. This may explain why controlling lower urinary tract function occurs so later in infancy, usually after walking and speaking. Taken together with anatomical and functional changes induced by age, this accounts for the high prevalence of lower urinary tract symptoms (LUTS) in both genders all over the world (Irwin et al., 2011).

LUTS are associated with great emotional burden to individuals (Engstrom et al., 2005) and substantial economic costs to society (Hu et al., 2003). As their prevalence increases with life expectancy, they are expected to be a major concern to physicians in the near future (Irwin et al., 2009). LUTS are generally divided into three groups: storage, voiding and postmicturition symptoms. Storage symptoms include increased urinary frequency, nocturia, urinary urgency, and urinary incontinence. Voiding symptoms include slow/weak stream, hesitancy, intermittency, straining and terminal dribble, whereas postmicturition symptoms consist of incomplete emptying and postmicturition dribble (Abrams et al., 2002).

According to the EPIC study, more than 62.5% of adult men and 66.6% of adult women report at least one urinary symptom throughout life (Irwin et al., 2006). Storage symptoms are the most prevalent and are more common in women (59.2% vs 51.3%) whereas voiding symptoms are more common in men (25.7% vs 19.5%)(Irwin et al., 2006). Storage symptoms have also higher impact on quality of life (Coyne et al., 2008; Peters et al., 1997) and are a more frequent cause of healthcare seeking behaviour (Apostolidis et al., 2012).

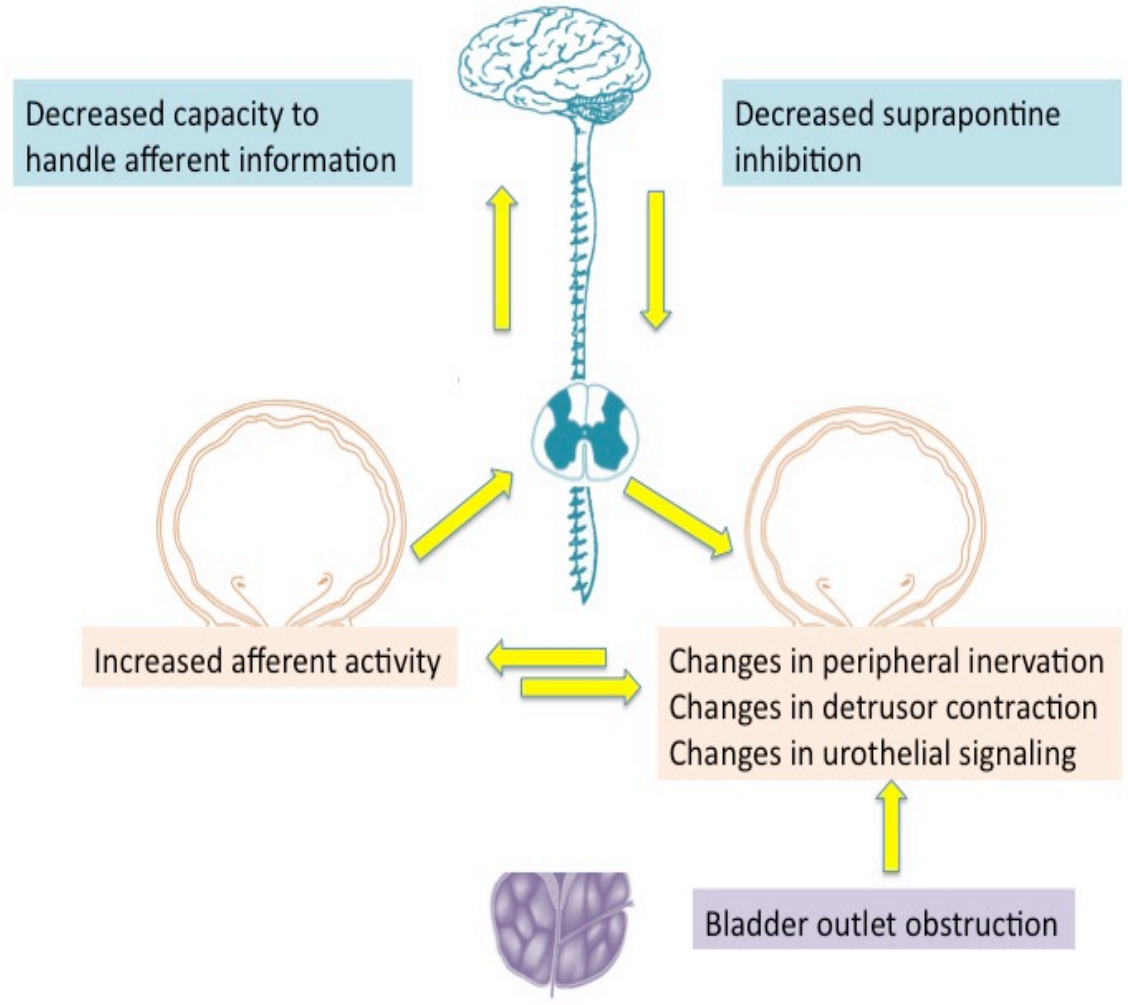


Figure 1. Central and peripheral mechanisms involved in the development of bladder dysfunctions.

1. 2 LOWER URINARY TRACT DYSFUNCTIONS- FOCUS ON THE BLADDER

The cause of LUTS is multifactorial but bladder dysfunction seems to be pivotal in its pathogenesis. Normal bladder function requires adequate control of the cerebral cortex, pons and spinal cord; intact autonomic and somatic nerves; and a normal lower urinary tract. Disorders in any of these structures my result in bladder dysfunction. Figure 1 portrays possible mechanisms that affect bladder activity and cause storage LUTS.

It is accepted that the bladder is capable of only a limited range of behaviours in response to disease and, for that reason, diverse pathologic

mechanisms may manifest as the same urodynamic abnormality. Furthermore several bladder dysfunctions may co-exist, for instance a patient with blader outlet obstruction (BOO) can exhibit either detrusor overactivity and detrusor underactivity (DU). For practical reasons, bladder dysfunctions will be further described according to the underlying urodynamic abnormality.

1.2.1 BLADDER OUTLET OBSTRUCTION

BOO is an urodynamic concept, characterised by increased detrusor pressure and low flow rate during voiding. BOO can be caused by a wide array of conditions, such as urethral strictures, urethral valves, female pelvic organ prolapse and urinary sphincter dysfunction. Yet, more commonly it is caused by benign prostatic hyperplasia (BPH). Traditionally BOO has been thought to be the cause of symptoms in patients with BPH and the relief of obstruction is the aim of most therapies designed to treat patients with BPH. In fact, BPH is a histological condition that causes benign prostatic enlargement that in some patients produce BOO by causing a physical compression of the urethra. However, the relation between LUTS, BOO and BPH is by far more complex. Firstly, the prostatic volume does not correlate linearly with the severity of BOO and LUTS (Eckhardt et al., 2001; Rosier & de la Rosette, 1995); and secondly, BOO can cause bladder dysfunction that clearly exacerbates the severity and nature of LUTS. This is a significant problem since around 30% of men older than 50 have moderate to severe LUTS (Boyle et al., 2003; Rosen et al., 2003), and it is estimated that roughly 50% of men with LUTS and BPH are indeed obstructed (Oelke et al., 2007).

In recent years increasing evidences place the bladder as the central organ in the pathogenesis of LUTS in BPH patients. BOO can induce two basic types of bladder dysfunction. On one hand, it can lead to detrusor overactivity and reduced bladder compliance that is associated with frequency and urgency symptoms. On the other hand, detrusor underactivity may occur later on in the course of the disease (Mirone et al., 2007), which cause further worsening of the voiding symptoms (see below).

At the tissue level, BOO produces several modifications in the bladder mucosa, detrusor smooth muscle and nerves as compensatory responses to overcome the high urethral resistance. One noticeable feature is bladder

hypertrophy; this is a constant finding both in animals and men with BOO (Michel & Barendrecht, 2008). In patients with BPH, bladder wall thickness measured by ultrasound correlates well with the severity of LUTS and obstruction (Oelke et al., 2007). Bladder hypertrophy results both from smooth muscle cells enlargement and extracellular matrix deposition. It has been shown that the obstructed detrusor has a higher collagen content (Inui et al., 1999; Mirone et al., 2004) and there seems to be a relation between collagen content and symptoms severity (Mirone et al., 2004).

Obstruction also leads to substantial changes in the neural control of the bladder. Several studies have shown significant bladder denervation in BOO patients comparing to controls (Chapple et al., 1992; Gosling, 1997). Since nerves are very sensitive to hypoxic damage, it has been hypothesized that postganglionic parasympathetic neurons could be damaged by the transient wall ischemia that occurs during voiding in obstructed bladders (Geloso & Levin, 1998). There is also evidence of a sensory dysfunction involving afferent C-fibers in BOO patients that could lead to detrusor overactivity. It has been shown in animal models that BOO induces an enlargement of afferent nerves in dorsal root ganglia (Steers et al., 1991) and an increase in the NGF content on the bladder tissue (Steers et al., 1991). It has also been reported that 71% of patients with BPH and BOO have a positive ice-water test suggesting a C-fiber hyperactivity usually inactive in normal adults (Chai et al., 1998). Accordingly, C-fiber desensitization with resiniferatoxin induced an improvement of storage LUTS in BPH patients (Dinis et al., 2004).

Bladder urothelium also changes with obstruction. Bladder epithelium from obstructed patients has been shown to have increased permeability to sodium and release higher amounts of adenosine triphosphate (ATP) (Araki et al., 2004; Silva et al., 2015). There have also been reported changes in the suburothelial interstitial cell network. In rat models of BOO, the population of these cells is increased and exhibit higher P2X3 receptor function (Kim et al., 2011; Li et al., 2013).

At present, current knowledge on the mechanisms underlying detrusor dysfunction after BOO is scant, and new ways to diagnose, predict, prevent and treat this condition are deemly needed.

1.2.2 DETRUSOR OVERACTIVITY

Detrusor overactivity is assumed to be the cause underlying the symptoms of overactive bladder (OAB). The International Continence Society defines OAB as a clinical syndrome based on the symptom of urinary urgency, with or without urinary incontinence and usually accompanied by frequency and nocturia, in the absence of infection or other obvious pathology (Abrams et al., 2002). It is estimated that 11-16% of men and 13-17% of women are affected (Irwin et al., 2006; Stewart et al., 2003). The impact of OAB on quality of life is significant, patients are predisposed to depression, sleep disturbance and falls, mainly in women with urge urinary incontinence (Brown et al., 2000; DuBeau et al., 1999; Dugan et al., 2000).

Detrusor overactivity is an urodynamic concept characterized by involuntary detrusor contractions during the filling phase. Detrusor overactivity may be further qualified according to cause, into neurogenic if there is a relevant neurologic condition or idiopathic when there is no defined cause. It is also possible to link detrusor overactivity to BOO. Indeed detrusor overactivity is present in about half to two thirds of patients with benign prostatic obstruction and disappears in almost two thirds of the patients after relief of obstruction by surgery (Abrams et al., 1979; de Nunzio et al., 2003). Similarly women with BOO have a significantly higher prevalence of detrusor overactivity compared to other women with LUTS but without BOO (Silva-Ramos et al., 2013a). However the exact mechanism by which obstruction induces detrusor overactivity is mostly unknown.

As shown in Figure 1 the pathogenesis of detrusor overactivity is multifactorial and poorly understood. For the sake of clarity, we can organize it into three different mechanisms:

- *Urothelial-suburothelial* mechanisms. Urothelium is more than a barrier. It has an higher metabolic activity and receptor density than the detrusor, allowing for an important neuromodulatory role (Hypolite et al., 1993). Modification on urothelial receptor function and release of signaling molecules impacts on the suburothelial interstitial cellular networking, which can originate involuntary detrusor contractions (Birder, 2006).

- *Myogenic* mechanisms. Changes in smooth muscle cells coupling and patchy denervation seen in obstructed detrusor samples can cause spontaneous contractions and enhanced propagation of excitatory signals between muscle cells.
- *Neurogenic* mechanisms. Detrusor overactivity may arise from several neurogenic mechanims. It can be originated via widespread nerve-mediated excitation of the detrusor muscle owed to the reduction of central inhibition. This mechanism promotes the intensification of spinal bladder reflexes and involuntary detrusor contractions. On the other hand, sensitization of C-fiber bladder afferent neurons can also trigger detrusor overactivity.

The relationship between OAB symptoms and the presence of detrusor overactivity in urodynamic studies is far from being perfect (Hashim & Abrams, 2006). Ultimately this may be due to two reasons:

- Symptoms are subjective and urgency patients report may not be due to detrusor overactivity.
- The sensitivity of urodynamic tests to diagnose detrusor overactivity is low. In fact, urodynamic studies test vesico-urethral function in very specific conditions and in a very limited time frame, which often does not mimic real life situations.

This renders the evaluation of patients' reported symptoms particularly challenging, hence the aphorism "the bladder is an unreliable witness" (Bates et al., 1970)

1.2.3 DETRUSOR UNDERACTIVITY

The International Continence Society defines detrusor underactivity (DU) as "a contraction of reduced strength and/or duration resulting in prolonged bladder emptying and/or a failure to achieve complete bladder emptying within a normal time span" (Abrams et al., 2002). Besides the somewhat ambiguous definition, clinical diagnose of DU is troubled with the need of a pressure flow study. This might partially explain why it is under-diagnosed, insufficiently investigated and poorly understood. In fact, we may be facing another "urologic iceberg". Prevalence estimates show that this is a fairly common urodynamic

finding, ranging from 9% to 23% in men under 50 years old (Osman et al., 2014) and increasing to 48% in men over 70 years old (Abarbanel & Marcus, 2007). In women prevalence is somewhat lower than in men, and ranges between 12% and 45% in elderly women (Osman et al., 2014). However, the diagnosis of underactive bladder is not so common in clinical practice.

There are few clues about the pathogenesis of DU, but it is accepted that is more than just a smooth muscle contractile dysfunction, both myogenic and neurogenic (afferent, central nervous system and efferent) factors may be involved. Multiple disorders and diseases may cause DU. These include, ageing, BOO, diabetes, spinal cord and cauda equina injuries, several neurologic disorders, amyloidosis and pelvic surgery (Juszczak & Drewa, 2016).

Clinically it is difficult to distinguish DU from other lower urinary tract dysfunctions. Voiding symptoms are quite prevalent in patients with DU, but these are indistinguishable from those associated with BOO, although symptoms of straining to void and decreased bladder sensation are more specific of DU (Gammie et al., 2015). Patients with chronic urinary retention due to DU, can also report storage symptoms such as frequency, incontinence and enuresis, being difficult to tell apart from patients with OAB. Furthermore, DU can coexist with BOO and detrusor overactivity rendering both diagnosis and treatment of these patients very challenging.

Therapeutic options for DU are limited. Theoretically, it is possible to envisage pharmacological targets to increase detrusor contractility. The approach may rely on enhancing both cholinergic and/or purinergic tonus in the detrusor.

1.3 THE PURINERGIC SYSTEM

In the 1970s when Burnstock formulated the purinergic neurotransmission hypothesis (Burnstock et al., 1972), the idea was received with tremendous scepticism, but it is now accepted that certain nerves use purine nucleotides as neurotransmitters. It is also recognised that ATP is not only released by nerves but also by non-neuronal cell types including the urothelial and interstitial cells. It mediates several biological processes such as synaptic transmission, nociception, ion transport, apoptosis, secretion and muscle contraction

(Burnstock, 2006). It is also implicit in the purinergic transmission hypothesis the presence of purinergic receptors. They were first divided in P1 receptors that respond to adenosine and P2 receptors for ATP, ADP and pyrimidine nucleotides (UTP and UDP). P2 receptors are further divided in two families: the P2X (P2X1-P2X7) ligand-gated ionotropic channel family and the P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) metabotropic G-protein coupled receptor family (Alexander et al., 2013a; Alexander et al., 2013b). P2X receptors are activated exclusively by ATP and respond more rapidly than P2Y receptors; the former act via ion channels and are more liable to desensitization (Burnstock, 2000; Ralevic & Burnstock, 1998). P1 receptors are also further divided into A_1, A_{2A}, A_{2B} and A_3, all of which are G protein-coupled receptors (Fredholm et al., 2011). The A_1 and A_3 receptors interact preferentially with members of the G_i protein family resulting in inhibition of adenylate cyclase and in the reduction of cyclic AMP (cAMP) production, whereas A_{2A} and A_{2B} receptors are coupled to G_s proteins and stimulate intracellular cAMP accumulation. All four receptors may also activate phospholipase C (PLC), resulting in inositol 1,4,5-trisphosphate (IP_3) production and increased intracellular Ca^{2+} mobilization (Klinger et al., 2002). Adenosine receptors can also stimulate mitogen-activated protein kinase cascades (Schulte & Fredholm, 2003).

One particularity of the purinergic system is that the extracellular metabolites of triphosphate nucleotides are also biologically active and can activate different receptors from the parent compound, which often counteract the initial signal. This gives the metabolic pathway a special importance. Extracellular nucleotides are hydrolyzed in a stepwise fashion into nucleosides by four main families of ectonucleotidases, both soluble and membrane-bound: ectonucleoside triphosphate diphosphohydrolases (NTPDases), nucleotide pyrophosphatase/phosphodiesterases (NPPs), alkaline phosphatases and ecto-5'-nucleotidase. Taking into consideration differences in the affinity of the ecto-enzymes for nucleotide substrates, the purinergic cascade is usually initiated by a ecto-nucleoside triphosphate diphosphohydrolase (ENTPDase;EC3.6.1.5) and is terminated by the ecto-5'-nucleotidase (CD73;EC3.1.3.5), which dephosphorylates nucleoside monophosphates into nucleosides. Adenosine is further converted to inosine by adenosine deaminase (ADA; EC3.5.4.4). As a result, extracellular adenosine concentrations are regulated by both ecto-5'

nucleotidase and ADA, acting together with a relatively efficient nucleoside uptake system (Figure 2).

Eight different NTPDases have been described. Four of these are typically cell-surface enzymes with an extracellular catalytic domain (NTPDase1,2,3,8). These enzymes differ in their substrate affinity and product formation. NTPDase 1 hydrolyses ATP and ADP equally well, NTPDases 3 and 8 have higher affinity for ATP, whereas NTPDase 2 is a preferential triphosphatase metabolizing almost exclusively nucleoside triphosphates.

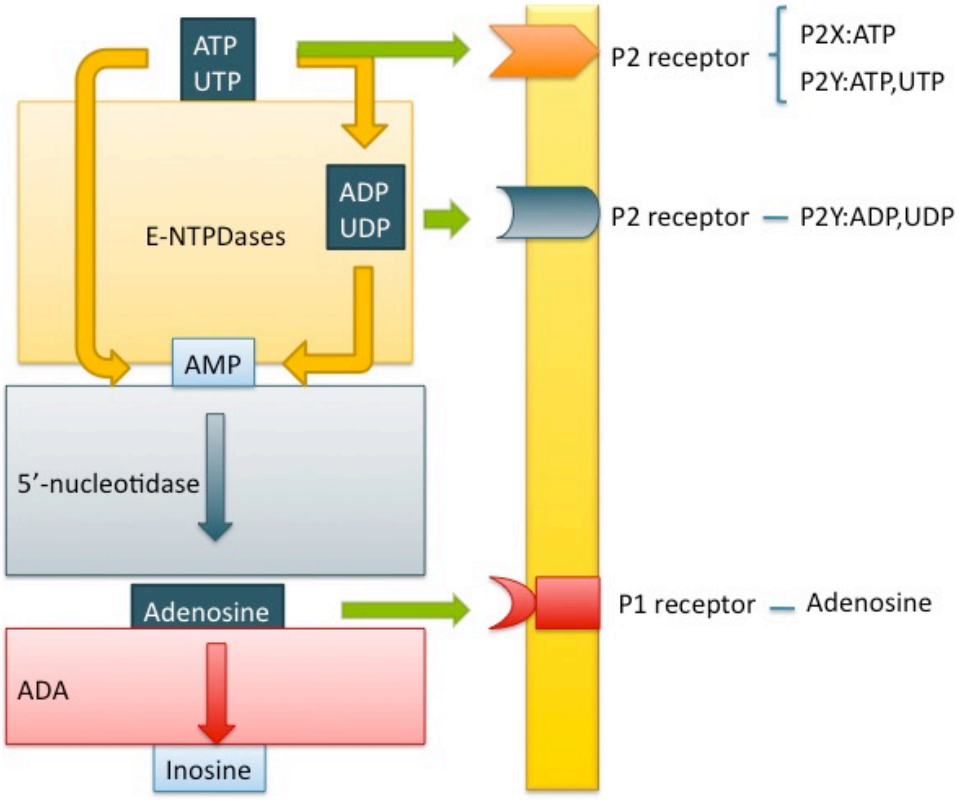


Figure 2. Extracellular catalytic cascade of nucleotides and potencial receptor activation.

Regarding product formation, NTPDase1 hydrolyzes ATP directly into AMP with transient production of minor quantities of ADP, thus bypassing P2Y receptors that are activated by nucleoside diphosphates. In contrast, the presence of NTPDase 2 results in the accumulation of ADP which is slowly dephosphorylated to AMP, thus promoting the activation of ADP-sensitive P2Y receptors ($P2Y_{1,\ 12,\ 13}$). NTPDases 3 and 8 exhibit an intermediate pattern of

product formation, leading to the transient formation of diphosphonucleosides with the simultaneous presence of triphosphonucleosides (Robson et al., 2006)

Only a single gene has been identified for ecto-5'-nucleotidase. This enzyme is responsible for most of adenosine formation from released adenine nucleotides (Zimmermann, 2000). Once produced, adenosine may undergo bidirectional translocation between cytoplasm and the extracellular fluid (through equilibrative or concentrative nucleoside transporters), deamination into inosine by adenosine deaminase (ADA, EC 3.5.4.4), and phosphorylation by intracellular adenosine kinase (EC 2.7.1.20) to generate AMP.

As for NPP1 and NPP3, they release nucleoside 5'-monophosphate from a variety of nucleotides, but intriguingly, their phosphorylated product (e.g. AMP) bind to NPPs with a higher affinity than substrates do, and thus inhibit catalysis (Stefan et al., 2005). Finally, the alkaline phosphatases are a family of abundant enzymes with broad range specificity, including dephosphorylation of ATP, ADP as well as AMP. The overall role of this family in purinergic signaling is, however, not that well investigated (Zimmermann, 2000).

1.3.1 ATP AND BLADDER

The content of this section was adapted from the review chapter:

Silva-Ramos M, Silva I, Ferreirinha F, Faria M, and Correia-de-Sá P (2016) Overactive Bladder (OAB): Is There a Place for Drugs Targeting the Purinergic Cascade? In: *Overactive Bladder (OAB): Prevalence, Risk Factors and Management.* Hauppauge NY; Nova Science Publishers, 2016.

Evidence for the role of ATP in bladder physiology, comes right from the beginning of the purinergic hypothesis formulated by Geoffrey Burnstock in the seventies. Indeed non-adrenergic and non-cholinergic (NANC) neurotransmission were first studied in the gut and in the urinary bladder (Burnstock, 1972; Burnstock et al., 1972). Since then, the knowledge of purinergic signalling in the lower urinary tract has expanded considerably. Purines are important regulators of normal bladder function, but also changes in purinergic signalling are associated with bladder dysfunction, particularly the overactivebladder. Although OAB is symptomatically complex, often idiopathic, it can be originated from multiple aetiologies. Mounting evidences indicate that

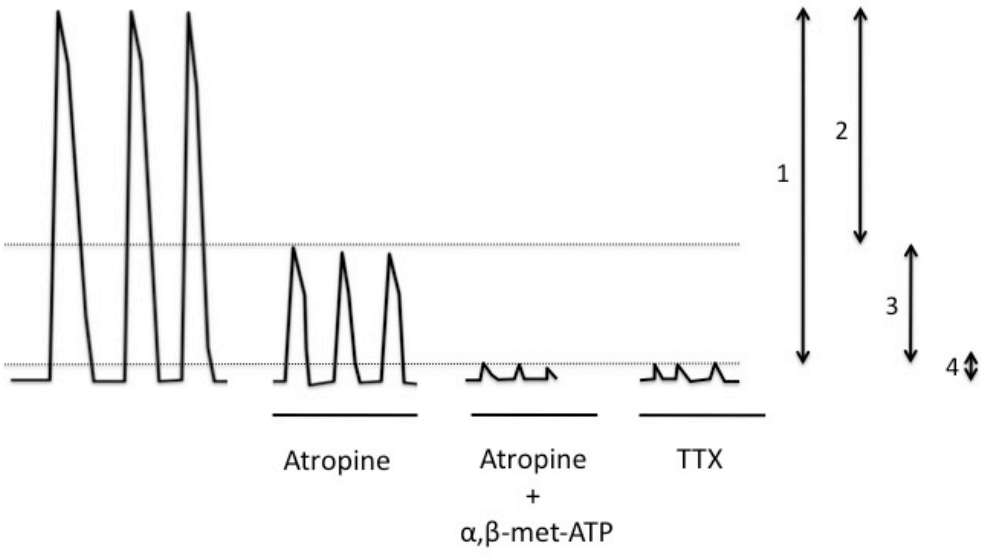
BOO and neurologic disorders are common causes of OAB and this is why much fundamental research on OAB comes from studying obstructed and neurogenic bladders.

PURINERGIC EFFERENT MECHANISMS

Pioneering evidences that NANC contractions are caused by ATP came from similarities between NANC and ATP-induced contractile activity and from the depression of NANC responses owed to tachyphylaxis produced by previous application of high ATP concentrations (Burnstock et al., 1972). In agreement with this, it was demonstrated that desensitization of P2 purinoceptors with the enzymatically stable ATP analogue, α,β-methyleneadenosine 5′-triphosphate (α,β-meATP), abolished nerve-induced NANC contractions of the urinary bladder (Brading & Williams, 1990). Direct evidences came later on, by measuring the release of ATP during stimulation of NANC nerves using the luciferin-luciferase bioluminescence assay (Burnstock et al., 1978b). *In vivo* experiments in rats demonstrated that electrical stimulation of the spinal cord triggered increases in intravesical pressure, which were antagonized by pre-treatment with α,β-meATP or pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS, a non-selective P2 receptor antagonist) (Hegde et al., 1998). Furthermore, cystometry experiments using non-anaesthetized rats showed that intra-arterial administration of ATP or α,β-meATP close to the bladder produced a dose dependent increase in bladder pressure (Igawa et al., 1993). Despite all these evidences, the study of the pos-junctional effect of ATP in the detrusor has been hampered by its metabolism into other biologically-active nucleotides with opposing effects and by P2 purinoceptors desensitization. Notwithstanding this, it is generally accepted that ATP released from efferent nerves cause bladder contractions in rodents via the activation of α,β-meATP-sensitive ionotropic P2X receptors, both in vitro and in vivo (Hegde et al., 1998; Lukacsko & Krell, 1982).

A slightly different scenario is observed in humans, where normal bladder contractions are mediated predominantly through the activation of muscarinic cholinergic receptors located in the detrusor smooth muscle with a very limited participation of ATP-sensitive P2 receptors. Human detrusor smooth muscle expresses muscarinic receptors of M_2 and M_3 subtypes, with the M_2 receptor

(66%) exceeding the M_3 receptor (33%) in number, but the latter being more active to cause smooth muscle contraction and voiding command under normal circumstances. The role of the M_2 receptor is still a matter of debate, even though it may contribute to bladder contraction in certain disease conditions (e.g. denervated bladder, neurogenic bladder dysfunction). Atropine-resistant contractions have been reported in the normal human detrusor, but these do not substantially contribute to bladder emptying under physiological conditions in contrast to that occurring in small laboratory animals (Ruggieri, 2006). The atropine-resistant purinergic component of the human bladder contraction may increase up to 40% in pathological conditions, such as OAB secondary to prostatic obstruction, interstitial cystitis, neurogenic bladders and aged bladders (reviewed in Burnstock, 2014). Under these circumstances, which resemble the normal function of the bladder in laboratory animals (See Figure 3), atropine-resistant contractions are attributed to ATP and may be due to (i) increased ATP release from nerves, (ii) impaired catabolism of ATP, and/or (iii) higher sensitivity of the detrusor smooth muscle to the nucleotide.



1. Nerve-mediated contraction
2. Cholinergic contraction
3. Atropine resistant-contraction
4. Direct muscle stimulated contraction

Figure 3. Scheme of nerve-mediated contraction magnitude, cholinergic and non-cholinergic components and residual direct muscle contraction. The contraction magnitude is indicated by the height of each bar.

Evidence for the first hypothesis is lacking; although there is evidence of increased ATP release with age, there are no reports of increased neuronal ATP release associated with specific urologic conditions (Yoshida et al., 2004).There is some support for the impaired catabolism option. In fact, it has been shown a decrease in ecto-ATPase activity in a mixed group of patients with detrusor overactivity and bladder outlet obstruction, thereby increasing tonic activation of P2 purinoceptors by endogenously produced ATP (Harvey et al., 2002). Concerning the sensitivity of the detrusor to ATP, there are conflicting results in the literature. In isolated detrusor myocytes, ATP evoked a similar increase in intracellular Ca^{2+} in both normal and overactive detrusor (Wu et al., 1999). On the other hand, ATP evokes more potent contractions in isolated detrusor samples from overactive or obstructed bladders compared with that from normal bladders (Harvey et al., 2002). Surprisingly the increase in ATP potency was not followed by its stable analogue, α,β-meATP, which evoked contractions with a similar magnitude in both groups (Harvey et al., 2002). The latter observations are consistent with the higher ATP bioavailability hypothesis due to deficient nucleotide catabolism. Notwithstanding this, a different sensitivity of the detrusor to ATP in diseased samples cannot be discarded since fast receptor desensitization induced by α,β-meATP might limit full detrusor contractions in overactive or obstructed bladder samples.

The recent availability of P2X receptor antagonists with subtype selectivity and of knockout mice allowed confirmation of the involvement of the P2X1 receptor in atropine-resistant contractions. In anesthetized rats the P2X1 receptor antagonist, RO116-6446, caused a significant attenuation of phasic isovolumetric contractions, without affecting volume and pressure thresholds (King et al., 2004). Moreover, ATP-induced contractions are completely absent in P2X1 receptor knockout mice (Vial & Evans, 2000). The P2X1 receptor has also been shown to be the dominant P2X receptor in the human detrusor using quantitative reverse transcription polymerase chain reaction (RT-PCR) (O'Reilly et al., 2001). Furthermore, immunohistochemistry studies showed that P2X1 receptor subunits are localized in the periphery of detrusor smooth muscle fibres (Elneil et al., 2001). Alltogether these data strongly suggest that the dominant receptor mediating purinergic contractions in the detrusor smooth muscle is the P2X1 receptor. Despite this, evidences for the role of the P2X1 receptor in the

pathophysiology of bladder syndromes are conflicting. Using RT-PCR, some authors reported no differences in P2X1 receptor transcripts among normal and obstructed bladder samples (Chua et al., 2007), while others showed increased amounts of these transcripts in obstructed samples (O'Reilly et al., 2001). Post-translational protein modifications may affect the amount of functional receptors in the plasma membrane and not always reflect gene transcription particularly under pathological conditions. These conflicting results were recently dismissed since we showed an increase in the P2X1 receptor protein immunoreactivity on detrusor smooth muscle cells from obstructed patients compared to control individuals, which fully supports the higher ATP potency detected in myographic recordings using bladder strips from these patients (Silva-Ramos et al., 2016a).

P2Y receptors may also have a role in mediating detrusor response to ATP stimulation. At least in laboratory animals, pharmacological studies show that P2Y may be involved in relaxation of pre-contracted detrusor (McMurray et al., 1998). On the other hand P2Y agonists like ADP and adenosine 5′-[β-thio]diphosphate(ADPβS) are capable of inducing detrusor smooth muscle contractions (Aronsson et al., 2010; McMurray et al., 1998; Palea et al., 1994; Suzuki & Kokubun, 1994; Yu et al., 2011). This effect was recently attributed to activation of the $P2Y_{12}$ receptor because it was blocked by the selective $P2Y_{12}$ receptor antagonist, PSB0739, but was unaffected by antagonists displaying affinity for other ADP-sensitive P2Y receptors, like $P2Y_1$ and $P2Y_{13}$ (Yu et al., 2011). However, the identified $P2Y_{12}$ receptor mediating ADP-induced bladder contractions has unique pharmacological properties because its effect on detrusor smooth muscle is temporally regulated by ectonucleotidases, both NTPDase1 and ecto-5'-nucleotidase, and by adenosine signalling. The crosstalk between $P2Y_{12}$ and adenosine receptors may be at the second messenger level since inhibition of the adenylate cyclase via $G_{\alpha i}$ provided by the $P2Y_{12}$ receptor activation may counteract the smooth muscle relaxation attributed to A_{2A}/A_{2B} receptors positively coupled to activation of this enzyme by $G_{\alpha s}$ proteins. The therapeutic significance of $P2Y_{12}$ in hemostasis and thrombosis has long been recognized due to the potent anti-thrombotic effects of thienopyridine compounds like ticlopidine and clopidogrel, which selectively inhibit $P2Y_{12}$ receptors in platelets. Given the recent data implicating the expression and function of $P2Y_{12}$

receptors in the detrusor smooth muscle contraction, these receptors may open new therapeutic avenues for the use of thienopyridine compounds in human bladder disorders and LUTS.

$P2Y_1$ receptor transcripts have been found in cells from the detrusor, although ADP-induced detrusor contractions were not inhibited by MRS2179. On the other hand, electrophysiological recordings in platelet-derived growth factor receptor-α-positive ($PDGFR\alpha^+$) interstitial cells in the detrusor muscle showed that ATP elicited large amplitude outward currents and hyperpolarization mediated by small conductance Ca^{2+}-activated K^+ (SK) channels sensitive to apamin. The $P2Y_1$ receptor agonist, MRS2365, mimicked the effect of ATP, and a $P2Y_1$ antagonist, MRS2500, inhibited ATP-activated SK currents (Lee et al., 2014). Localization of purinergic inhibitory effects in $PDGFR\alpha^+$ cells may provide the basis for a novel neural regulatory mechanism for the stabilization of bladder excitability during the filling phase. These authors observed that during extended neural stimulation of bladder muscles, the sustained component of contraction is significantly reduced by $P2Y_1$-mediated purinergic regulation. Thus, adenine nucleotides released from autonomic neurons may have dual, temporally-distinctive, actions in micturition: activation of detrusor contraction via P2X1-receptors in smooth muscle cells during voiding and stabilization of membrane potential and excitability during bladder filling through the activation of $P2Y_1$ receptors in $PDGFR\alpha^+$ cells. The purinergic inhibitory input mediated by $P2Y_1$ receptors may potentiate detrusor relaxation during urine storage caused by activation of adrenergic β_3 receptors (Afeli et al., 2012; Brown et al., 2008). This synergism may be pathophysiologically relevant considering that discrete inhibitory neurons are extremely rare in the bladder (Lee et al., 2014).

In contrast to the compelling evidence for the extracellular signaling role of ATP and related adenine nucleotides, the hypothesis that uracil nucleotides exert autocrine/paracrine roles has only recently gained experimental support. Uridine diphosphate (UDP) sensitive $P2Y_6$ receptors have been involved in the generation of large spontaneous contractions and propagating waves of intracellular Ca^{2+} and membrane depolarization originating in suburothelial myofibroblasts and spreading to the detrusor smooth muscle in rats submitted to spinal cord transaction (Fry et al., 2012). Furthermore, using myographic recordings it has been demonstrated that UDP, acting on $P2Y_6$ receptors,

interplays with P2X1 receptors in a synergistic manner to increase bladder smooth muscle tone (Yu et al., 2013). We confirmed that UDP increased the frequency and the amplitude of spontaneous detrusor contractions in the isolated rat bladder when the nucleotide was applied in high concentrations (300 μM) outside the bladder wall, but not when it was infused into the bladder lumen (Carneiro et al., 2014). However, increments of spontaneous bladder twitching caused by UDP (300 μM) were minimal (<10%) when compared to muscarinic and P2X1 receptors activation, respectively with oxotremorine (30 μM) and α,β-meATP (30 μM). A significant discrepancy was also observed between the lack of effect of intraluminal UDP in the isolated bladder and the increase in the voiding frequency when the nucleotide was infused into the bladder lumen in anaesthetized rats. Overall, these results indicate that direct activation of smooth muscle via co-expressed $P2Y_6$ and P2X1 receptors might represent a minor component of the urodynamic response to UDP that is mediated predominantly by the sequential activation of urothelial $P2Y_6$ receptors and ATP release resulting in stimulation of urothelial/sub-urothelial P2X3 receptors in the rat (Carneiro et al., 2014; Timóteo et al., 2014) and in humans (Silva et al., 2015). In addition, to the well-characterized post-junctional effect of ATP, the nucleotide released at the synaptic cleft, can act on prejunctional P2 receptors, modulating other transmitters release (Duarte-Araújo et al., 2009; Sperlágh & Vizi, 1991). This effect has been shown in rat and porcine detrusor; where, ATP was able to enhance parasympathethic motor drive through pre-synaptic P2X3 receptors (D'Agostino et al., 2012; King et al., 1997). Conceivably, this prejunctional modulatory effect of ATP also exists in the human bladder, although evidence has been lacking till now.

PURINERGIC SIGNALING DURING BLADDER FILLING

Most afferent nerves conveying sensitive information from the urinary bladder to the spinal cord originate in dorsal root ganglia. In the dorsal horn of the spinal cord, information received enters (1) via ascending nerve afferents, giving rise to conscious perception of bladder sensations, or (2) via interneurons, affecting motor pathways. At the bladder wall, C-fibre axons possess endings in the suburothelial layer and may also penetrate the urothelium (Birder et al., 2010). As the urothelium responds to mechanical and chemical stimuli, and

transmits information to underlying nervous and muscular systems, it can be regarded as a functional transduction unit playing a pivotal role in generating afferent signals to bladder intrinsic relay circuits and to control centres in the CNS.

The urothelium is a transitional epithelium composed of three to five cell layers: a basal layer attached to the basement membrane, an apical layer with large umbrella cells and an intermediate layer. The suburothelium is composed of nerves, interstitial cells, blood vessels and connective tissue; it closely interacts with the urothelial layer above, the detrusor layer below and acts as a functional unit. The umbrella cell layer has the ability to incorporate additional membrane into the luminal surface as it is stretched, increasing the surface area during bladder filling. This is done by fusion and exocytosis of a subapical pool of discoidal/fusiform-shaped vesicles (Truschel et al., 2002). Upon voiding, the mucosa refolds, and the membrane added to the apical surface of the umbrella cells is thought to be recovered by endocytosis. Besides changes in membrane capacity, pressure-induced membrane traffic in apical urothelial cells can dramatically change drugs targeting to membrane-bound receptors and their coupling to downstream signalling cascades. This leads to dynamic modifications of urothelial signals in a moment-to-moment basis in order to accommodate urine storage and/or trigger bladder emptying. However, little is known about the events underlying these changes in both physiological and pathological conditions.

ATP was the first transmitter shown to be released directly by the urothelium (Ferguson et al., 1997). The release of the nucleotide can be triggered both by chemical stimuli and by stretching the uroepithelium during bladder filling. Non-neuronal release of ATP plays an important role as an autocrine and/or paracrine signalling molecule. Once in the suburothelial layer, ATP may act on vimentin-positive interstitial cells and on afferent nerve fibres conveying sensory information to the CNS through the activation of P2X2 and P2X3 receptors, respectively (Silva et al., 2015). Immunohistochemistry experiments have shown immunoreactivity against the P2X3 receptor on nerve fibres in the lamina propria, urothelium and detrusor muscle (Ford & Cockayne, 2011). Mice lacking P2X3 receptor exhibited reduced inflammatory pain, prolonged voiding intervals and higher voided volumes (Cockayne et al., 2000). Experiments using an in vitro bladder-pelvic nerve preparation, show that prolonged exposure to a

desensitizing dose of α,β-meATP significantly reduces the activity of mechanosensitive pelvic nerve afferents in response to bladder distension (Namasivayam et al., 1999). Likewise, intravesical applications of ATP induce rapid excitation of bladder afferents, which was counteracted by PPADS and 2′,3′-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) (Vlaskovska et al., 2001). Similarly, P2X3 knockout mice exhibited a diminished response to bladder distension and to instillation of P2X receptor agonists manifested by delayed bladder emptying and increased threshold of activation of sensory afferents when compared to wild-type mice (Vlaskovska et al., 2001). A sensory role for the P2X2 receptor subtype has also been revealed using P2X2 and P2X2/P2X3 knockout mice (Cockayne et al., 2005).

The mechanisms underlying ATP release from urothelial cells is a matter of debate in the literature (Sui et al., 2014). ATP can be released from both surfaces of isolated uroepithelium through multiple mechanisms, including vesicular excocytosis, conductance of ATP through ABC or nucleoside transporters or translocation across hemichannels containing either connexins or pannexins. ATP released from the uroepithelium or surrounding tissue may act as a trigger for discoidal/fusiform vesicle exocytosis and membrane recovery through signal at uroepithelial P2 receptors (Wang et al., 2005). Several studies using RT-PCR, Western blotting and immunocytochemistry techniques have demonstrated that the urothelium expresses almost all purinoceptor subtypes. These include all ionotropic P2X receptor subunits and metabotropic $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (Carneiro et al., 2014; Chopra et al., 2008; Elneil et al., 2001; Lee et al., 2000; Shabir et al., 2013; Vial & Evans, 2000). Cooperation between metabotropic $P2Y_6$ and ionotropic P2X3 and/or P2X2/3 receptor subunits contributes to promote ATP and ACh release from non-neuronal sources in healthy human urothelium, but the positive feedback mechanism operated by released ATP through fast desensitizing P2X3 and/or P2X2/3 receptors is impaired in obstructed patients, without much affecting the $P2Y_6$ mediated facilitation (Silva et al., 2015). Our group has previously shown that activation of the $P2Y_6$ receptor increases the voiding frequency in anesthetized rats indirectly by releasing ATP from the urothelium via pannexin-1 hemichannels (Timóteo et al., 2014) and subsequent activation of P2X3 receptors on suburothelial nerve afferents. These results suggest that down-modulation of the

release of signalling molecules, such as ATP and ACh, from the bladder uroepithelium with selective $P2Y_6$ receptor antagonists may constitute a novel strategy to control hyperactivity of sensory nerves and persistent storage symptoms in patients with BOO.

Once released from the urothelium, ATP is rapidly dephosphorylated by membrane-bound ectonucleotidases. The four members of the NTPDase family, as well as 5′-nucleotidase, are expressed in the mouse bladder, as demonstrated by RT-PCR. However, immunolocalization studies failed to detect NTPDase1,2 and 5'-nucleotidase on rat urothelium (Yu et al., 2011). Notwithstanding this, adenosine has been shown to negatively modulate stretch-induced ATP release from apical umbrella cells through the activation of A_1 receptors (Dunning-Davies et al., 2013). Taking into consideration that adenosine levels at the luminal site of the urothelium are 10-fold below those found beneath the basal layer, one may hypothesize that adenosine A_1 receptor-mediated inhibition of ATP release plays a minor (if any) role in physiological conditions. Besides mechanical, chemical and purinergic stimulation, other compounds can elicit ATP release from urothelial cells. Notably, ACh has an important role in modulating this process. All 5 muscarinic receptors are present in urothelial cells. Stimulation of these receptors can trigger ATP release from urothelial cells in culture (Kullmann et al., 2008). Likewise, intravesical infusion of high doses of the muscarinic agonist oxotremorine increased the voiding frequency in anesthetized rats, an effect that was blunted by blocking P2X receptors with PPADS applied into to bladder lumen (Kullmann et al., 2008). Carbachol and oxotremorine also favored the release of ATP from the human urothelium and the effects of these drugs were abolished by the muscarinic M_2 receptor antagonist, methoctramine (Sui et al., 2014). These results suggest that activation of muscarinic receptors located near the luminal surface of the bladder affects the voiding function by mechanisms involving ATP release, thus strengthening the concepts that antimuscarinic drugs used to treat OAB syndromes exert their effect at least in part through actions in the urothelium.

In the suburothelial layer, the recently discovered interstitial cells have become an exciting focus of research (Wiseman et al., 2003). These cells form a syncytium through extensive coupling via connexin 43-containing gap junctions in a similar manner to that described in the gastrointestinal tract. Through this

arrangement signals initiated in a group of interstitial cells can travel considerable distances in the suburotelium and its information conveyed above to the urothelium and below to the detrusor smooth muscle layer. These cells are in close proximity to afferent nerves and elicit excitatory responses mediated by both purines and pyrimidines, such as ATP, ADP, UTP and UDP, via several P2Y receptor subtypes (Fry et al., 2007), including the most expressed $P2Y_6$ receptor (Sui et al., 2006).

ATP-MEDIATED SIGNALS IN PATIENTS WITH BLADDER DYSFUNCTION

Changes observed in the bladder of patients with BOO are very similar to those observed in OAB patients. For that reason they are described together in the following section.

Most OAB patients, excluding some neurogenic cases, report an increase in bladder sensation. Whether this is a cause or a consequence of OAB remains to be elucidated. Anyway, there is a considerable amount of evidence showing that afferent inputs are increased in these patients.

The urothelium from patients with neurogenic or idiopathic detrusor overactivity, as well as with BOO, release more ATP than urothelial strips from control individuals (Kumar et al., 2010; Silva et al., 2015; Sun et al., 2002). Although the basal release of ATP from both neurogenic and control bladders was similar, it reached values that were 20-fold higher the control amounts in bladders from patients with idiopathic OAB, indicating different pathophysiological mechanisms underlying the two disease conditions (Kumar et al., 2010). While in neurogenic OAB the defect is predominantly in the central control of the micturition, in idiopathic OAB the urothelium may play a dominant role in its pathogenesis. Likewise, in patients with BOO, both stretch and electric field stimulation (EFS) can trigger the release of ATP from the urothelium (Silva et al., 2015; Sun et al., 2002). EFS-induced ATP release from the urothelium was 5-fold higher in BOO patients compared to control; this difference can be attributed, at least partially, to an increase in the half-degradation time of ATP by ectonucleotidases confirming that these patients exhibit enhanced purinergic tone.

Moreover, patients with neurogenic detrusor overactivity possess higher P2X3 receptor amounts in suburothelial afferent sensory neurons compared to

control individuals (Brady et al., 2004). It was also observed that the number of suburothelial sensory nerves is increased in patients with idiopathic detrusor overactivity (Moore et al., 1992; Smet et al., 1997). Notably, it was demonstrated that responders to the transient receptor potential cation channel subfamily V member 1 (TRPV1) activator, resiniferatoxin, exhibit reduced expression of the P2X3 receptor after treatment, but this change was not observed in non-responders (Brady et al., 2004). The same group studied the effects of botulinum toxin A injection on P2X3 and TRPV1 receptors expression in patients with neurogenic and idiopathic detrusor overactivity; they found a decrease in the expression of both receptors in suburothelial nerve fibers after injection of the toxin (Apostolidis et al., 2005). Taken together these data indicate a role for ionotropic P2X3 receptors in the pathophysiology of detrusor overactivity.

Interestingly, the population of suburothelial interstitial cells is also increased in OAB patients (Roosen et al., 2009) and this is accompanied by increased expression of P2X2 receptor subunits demonstrated by immunofluorescence and Western blot analysis in female patients with OAB compared to controls (Meng et al., 2015). However, the same was not observed in male patients with BOO due to BPH (Silva et al., 2015). The immunoreactivity against P2X2 and P2X3 ionotropic receptor subunits localized respectively in vimentin-positive interstitial cells and suburothelial nerve afferents, as well as in the urothelium, almost disappeared in BPH patients submitted to surgery to relief BOO. Disappearance of the P2X2 immunostaining was not detected in suburothelial blood vessels from BPH patients. In contrast to the partial loss of ionotropic P2X receptors staining, immunoreactivity against the metabotropic $P2Y_6$ receptor was conserved in the urothelium of BPH patients compared to healthy controls. This receptor is abundantly expressed throughout the urothelial layer (including the more superficial umbrella cells), in suburothelial interstitial cells and in the detrusor smooth muscle; interestingly, the $P2Y_6$ staining accompanied the increase in the population of suburothelial interstitial cells observed in patients with BOO due BPH (Silva et al., 2015). These findings denote different purinoceptor arrangements depending on the underlying pathophysiological mechanism of OAB.

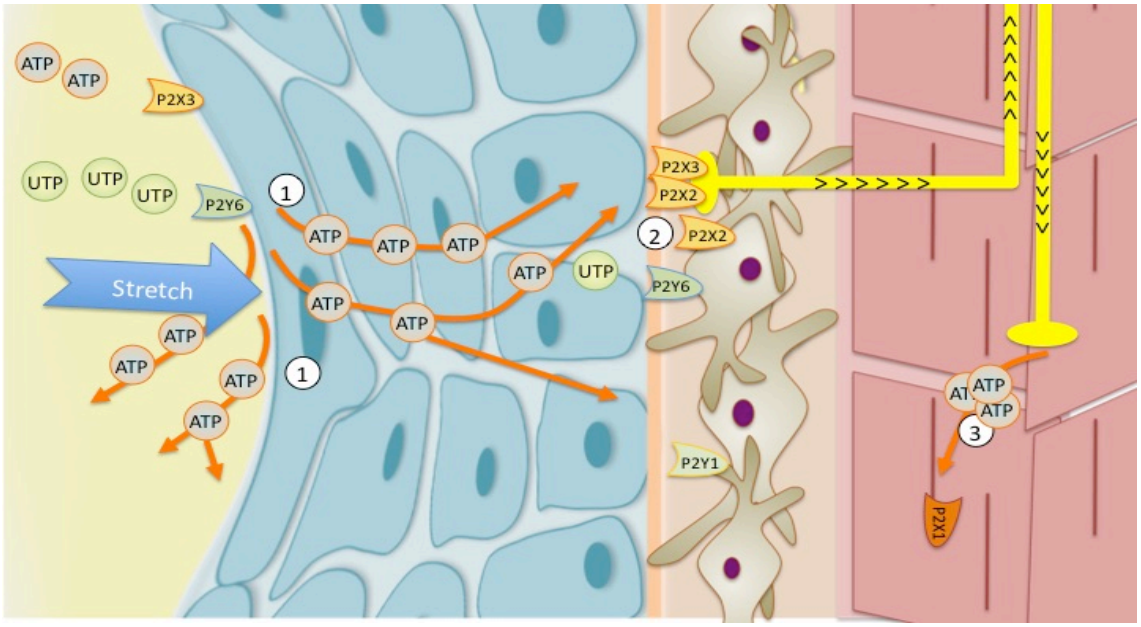


Figure 4. Mechanisms underlying enhanced purinergic tone in OAB. (1) The urothelium of OAB patients releases higher amounts of ATP; involvement of $P2Y_6$ receptors activation and ATP-induced ATP release operated by P2X3 and/or P2X2/3 receptors; (2) Surplus ATP released from the uroepithelium activates suburothelial P2X3 receptors on sensory nerve afferents and P2X2 receptors on interstitial cells leading to increased sensation during bladder filling.(3) Atropine-resistant purinergic contractile responses are also potentiated in the detrusor of OAB patients, which may be due to overexpression of P2X1 receptors and/or deficient ATP catabolism.

PURINERGIC CONTROL OF CENTRAL BLADDER REFLEXES

Several disorders of the CNS are known to cause OAB. CNS disease conditions are associated with detrusor overactivity , for instance, cortical lesions secondary to cerebral vascular accidents, multiple sclerosis lesions in the pons and traumatic spinal cord injuries. There is, however, a gap in our knowledge about the role of purines in bladder overactivity secondary to CNS diseases. Nowadays, the role of ATP as a fast synaptic neurotransmitter is by far best characterized at peripheral neuro-effector junctions than in the CNS (Khakh & North, 2012). Nonetheless, activation of P2 receptors on two important CNS areas controlling micturition reflexes (peri-aqueductal grey matter and Barrington nucleus) triggered increases in bladder pressure and efferent pelvic nerve activity, suggesting a role in modulating parasympathetic bladder innervation (Rocha et al., 2001).

At the spinal cord level, there is also evidence of ATP signaling mediating bladder reflexes. ATP mediated responses may be partially responsible for

detrusor overactivity induced by acetic acid, acrolein or cyclosphophamide in the rat (Masuda H et al., 2005). Involvement of ionotropic P2X3 and P2X2/3 receptor subunits have been claimed, since intrathecal administration of the potent and selective antagonist of these receptors, AF-792, produced long lasting inhibition of micturition reflex contractions (Khakh & North, 2012). These data support a role for presynaptic P2X3 and P2X2/3 receptors in facilitating the micturition reflex at the spinal cord level. Following spinal cord injury, the amount of ATP released in the damaged tissue increases dramatically (Peng et al., 2009), as well as the expression of P2X2 and P2X3 receptor subunits increase in the bladder (Brady et al., 2004; Pannek et al., 2009).

RELEVANCE OF ATP IN CLINICAL PRACTICE

In the clinical setting diagnosing OAB is not always an easy task. Symptoms are subjective, patients do not always mean what they say and clinicians do not always get what patients mean. Therefore, there is a clinical need for a simple non-invasive test that could help diagnosis of OAB. In the last few years several urinary biomarkers for OAB have been investigated and proposed (Antunes-Lopes et al., 2011). Since these patients exhibit an higher purinergic tone, identifying this may help diagnosis and better characterize these patients.

Targeting the purinergic system also seems a reasonable option for the treatment of several lower urinary tract dysfunctions. In fact, antimuscarinic drugs frequently used as a first-line therapeutic option for OAB lose efficacy with the time of treatment (Sears et al., 2010). Long-term exposure to anticholinergics is associated with downregulation of the postjunctional response to carbachol and with an upregulation of purinergic responses to ATP in experimental rats (Uvin et al., 2013), leading us to hypothesize that association with P2 receptor antagonists might be appropriate.

Novel, potent and orally-active P2X3 antagonists have been developed and tested in lower urinary tract dysfunctions (Gever et al., 2010). The selective P2X3 antagonist, A317491, improved bladder function parameters in a rat model of spinal cord injury and in cyclophosphamide-induced cystitis; this drug effectively inhibited non-micturition contractions, increased inter-contraction

intervals and bladder capacity (Ito et al., 2008; Lu et al., 2007). Likewise, AF-353 diminished the frequency of non-voiding contractions in spinal cord injured rats (Munoz et al., 2012). P2X3 antagonists, like AF-219, are being tested in clinical trials for chronic cough and interstitial cystitis/bladder pain syndrome (Abdulqawi et al., 2015; Moldwin R et al., 2015). A phase 2 study recently presented, showed that AF-219 improved pain and related symptoms in patients with interstitial cystitis/bladder pain syndrome compared to placebo, with minor adverse events (Moldwin R et al., 2015). Figure 4 shows the rationale for using selective P2X3 (or P2X2/3) antagonists in OAB syndromes, which may derive from their relevant role in urothelium-mediated pressure transducer signaling pathway, involving excessive ATP-induced ATP release from uroepithelial cells and activation of suburothelial nerve afferents and interstitial cells by non-neuronal released ATP.

Another interesting finding from our group suggests that UDP-sensitive $P2Y_6$ receptors activation triggers the release of ATP from urothelial cells via pannexin-1 hemichannels (Carneiro et al., 2014; Timóteo et al., 2014), which results in increased release of the nucleotide in BOO patients (Silva et al., 2015). Thus, blocking the $P2Y_6$ receptor in the urothelium may constitute a novel therapeutic approach to control bladder overactivity and persistent storage symptoms in patients with BOO.

Botulinum toxin A (BoNT-A) is clinically used to treat both neurogenic and idiopathic bladder overactivity when anti-muscarinic drugs fail (Cruz et al., 2011; Pinto et al., 2010). The actions of BoNT-A are not restricted to suppression of ACh and norepinephrine release from autonomic nerves in the rat bladder, but, interestingly, it also diminishes the release of ATP (Khera et al., 2004; Smith et al., 2003). Reduction of ATP release by BoNT-A may decrease the activation of sensory nerve afferents induced by bladder irritation with acetic acid (Chuang et al., 2004). Therefore, it appears that the clinical efficacy of BoNT-A relies not only on the blockade of transmitter release from efferent nerves, but also on its action on urothelial sensory mechanisms.

All together, these data support a crucial role of ATP and its derivatives in the pathophysiology of OAB and foresee future drugs targeting the purinergic cascade, including ATP release sites, ectonuleotidases, and purinoceptors, for the treatment of OAB syndromes.

1.3.2 ADENOSINE AND BLADDER

The content of this section was adapted from the review chapter:

Silva-Ramos M, Silva I, Faria M, Magalhães-Cardoso MT, Correia-de-Sá P: Urinary bladder disorders: Is adenosine friend or foe? In: *Adenosine receptors. Pharmacology, functions and therapeutic aspects*. Hauppauge NY; Nova Science Publishers, 2014.

In the previous section we highlighted findings demonstrating that ATP (1) is a co-transmitter with acetylcholine (ACh) in parasympathetic nerves regulating bladder contraction (Burnstock et al., 1972), (2) activates afferent nerves during bladder filling transmitting both normal and abnormal sensations like urgency and pain (Birder & Andersson, 2013; Ferguson et al., 1997; Wang et al., 2005), and (3) participates in the control of bladder reflexes at the CNS (Rocha et al., 2001). However, the effects of the ATP metabolite, adenosine, has been less studied. Initial reports on the effect of adenosine in the lower urinary tract are from the nineteen seventies and coincided with Burnstock's group attempt to prove that ATP was the non-cholinergic non-adrenergic neurotransmitter released from nerves supplying the urinary bladder (Burnstock et al., 1978b; Burnstock et al., 1972). They managed to show that the breakdown product of ATP, adenosine, caused a decrease in both the tone and the spontaneous activity of the urinary bladder (Burnstock et al., 1978a).

Adenosine is a ubiquitously produced nucleoside that participates in the normal function of the cardiovascular, respiratory, renal, gastrointestinal, neuronal, musculoskeletal and immune systems where it regulates diverse phenomena including neuronal excitability, vasodilation, smooth muscle relaxation, protection against ischemic and inflammatory insults, and the release of vasoactive and neuroactive substances. Adenosine is generated within cells from the hydrolysis of *S*-adenosyl-L-homocysteine in parallel to its formation from the catabolism of adenine nucleotides, ATP, ADP, AMP or cAMP, via nucleotidases, which exist both at the intracellular and the extracellular space (Fredholm et al., 2001; Jackson & Raghvendra, 2004). Once produced, adenosine may undergo bidirectional translocation between cytoplasm and the extracellular fluid (through equilibrative or concentrative nucleoside transporters),

deamination into inosine by ADA (EC 3.5.4.4), and phosphorylation by intracellular adenosine kinase (EC 2.7.1.20) to generate AMP. The way adenosine builds its influence to control cells communication depends on the extracellular concentration of the nucleoside, which is achieved by balancing extracellular adenosine generation and inactivation mechanisms (cellular uptake and/or extracellular deamination) (Correia-de-Sá & Ribeiro, 1996; Goncalves & Queiroz, 1993). Although adenosine transport may be faster than its subsequent intracellular metabolism (for a review see Geiger, 1991), numerous studies have shown that phosphorylation of adenosine by adenosine kinase is the primary metabolic pathway regulating both intra- and extracellular levels of the nucleoside (Arch & Newsholme, 1978b; Lloyd & Fredholm, 1995). In the urinary bladder, parallel formation of adenosine from the hydrolysis of ATP released from stimulated neuronal and non-neuronal cells (e.g. urothelial cells, interstitial cells, fibroblast-like cells, smooth muscle fibers) is also possible. Therefore, it is conceivable that adenosine contributes to an overall homeostatic role on bladder activity during instability conditions (*e.g.* inflammation, hypoxic/ischemic insults, outlet obstruction) when endogenous adenosine becomes elevated. Extracellular inactivation may restrict adenosine actions to the release/production sites and may limit diffusion of the exogenously added nucleoside towards the active zones (see e.g. Correia-de-Sá et al., 2006; Duarte-Araújo et al., 2004).

Once in the extracellular milieu, adenosine may interact with membrane-bound P1 receptors (Fredholm et al., 2001). In humans, the A_3, A_1 and A_{2A} receptor subtypes are activated by submicromolar concentrations of adenosine (0.29 μM, 0.31 μM, and 0.73 μM, respectively) while the adenosine A_{2B} receptor is activated at much higher concentrations of the nucleoside (23.5 μM) (Fredholm et al., 2001). This pattern differs significantly from that found in other mammals where the A_3 receptor has low affinity for adenosine. These findings suggest that the human adenosine A_1, A_{2A} and A_3 subtypes respond to physiological concentrations of adenosine (between 30 and 300 nM), while the A_{2B} receptor subtype requires higher concentrations of the agonist for activation which may rise from 10 to 1000 fold in pathological conditions.

ADENOSINE RECEPTORS EXPRESSION IN THE URINARY BLADDER

First studies aiming at characterizing pharmacologically P1 receptors in the urinary bladder were performed in mice and supported the role of both pre-synaptic (via A_1 receptors) and post-synaptic (via A_2 receptors) effects (Acevedo et al., 1992; Nicholls et al., 1992). The role of low affinity A_{2B} and A_3 receptor subtypes has been more difficult to characterize (see below). Paradoxically, RT-PCR analysis has detected all four adenosine receptor subtypes in the rat urinary bladder: A_{2A} and A_{2B} being more expressed than A_1, whereas A_3 was the least expressed (Dixon et al., 1996). A similar pattern was detected for the mRNA expression of adenosine receptors in the human bladder epithelial carcinoma T24 cell line (Phelps et al., 2006). Another study in female rats confirmed the predominance of the A_{2B} receptor mRNA in the urinary bladder and reported increases in P1 receptor mRNA with age (Owen et al., 2012). Because PCR results do not necessarily correlate with the presence of functional adenosine receptors displayed on the cell surface, further characterization of these data by western blot analysis and functional assays are highly recommended.

Using western blot analysis and immunofluorescence localization, Yu and col. detected the expression of all four types of adenosine receptors in the bladder urothelium of female Sprague-Dawley rats (Yu et al., 2006). The A_1 receptors were predominantly localized to the apical membrane of umbrella cells, whereas A_{2A}, A_{2B} and A_3 receptors were localized intracellularly or on the basolateral membrane of umbrella cells and the plasma membrane of underlying cell layers. Signals for the A_1 receptor were also observed in the underlying submucosal connective tissue, but suburothelial tissue elements (possibly connective cells, myofibroblasts or blood vessels) were more strongly labeled with the A_{2A} antibody. Although information regarding location of adenosine receptors on the detrusor is still scant, there are reports suggesting that A_{2B} receptors are abundantly expressed in rat bladder smooth muscle (Stehle et al., 1992; Yu et al., 2006). In addition, there are pharmacological evidences for the presence of A_1, A_{2A} and A_3 receptors on detrusor muscle, often with conflicting results depending on the species and experimental settings (Gopalakrishnan et al., 2002; Vesela et al., 2011; Yang et al., 2000). Surprisingly, there is little data

characterizing the adenosine receptor subtype(s) involved in the human urinary bladder function.

EFFECTS OF ADENOSINE IN THE UROTHELIUM

There are innumerous studies demonstrating that the urothelium can release ATP in response to stretch and chemical stimuli, in animals and in humans (Ferguson et al., 1997; Timóteo et al., 2014; Wang et al., 2003), but it is unknown whether this contributes to the high concentrations of adenosine found in urine (Heyne et al., 2004; Vidotto et al., 2003). However, it has been demonstrated that about 50% of the extracellular adenosine available is the result of the break-down of ATP (Smith & Lu, 1991) which is currently regarded to be the most important route of generating extracellular adenosine (Zhang & Xia, 2012). *In vitro* degradation studies of ATP in a guinea-pig detrusor strip organ bath setup showed that ATP was rapidly broken down within minutes to form mainly, but not exclusively, adenosine (Cusack & Hourani, 1984). Magnesium-dependent adenosine triphosphatase (Mg^{2+}-ATPase) as well as 5′-nucleotidase and alkaline phosphatase were identified in epithelial cells of the rat urinary bladder (Zhang et al., 1991). The four members of the NTPDase family, as well as 5′-nucleotidase, are expressed in the mouse bladder, as demonstrated by RT-PCR (Yu et al., 2011). The relative contribution of distinct ectonucleotidases to the modulation of purinergic signalling depends on differential tissue distribution, regulation of cell expression, targeting to specific membrane domains, but also on substrate preference and availability. This might explain the different kinetics of ATP metabolism and adenosine accumulation between luminal and serosal sides of the urothelium (Lewis & Lewis, 2006). Ussing chamber experiments with the rabbit urothelium showed that adenosine concentration rises in the incubation fluid by stretching the tissue, providing that the experiments are done in the presence of iodotubercidin (an inhibitor of adenosine kinase) and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, an inhibitor of ADA) (Prakasam et al., 2012; Yu et al., 2006); the highest concentration of adenosine was recorded at the serosal surface of the urothelium, with concentrations approaching 200 nM. Previous studies from the same group showed that increased pressure stimulates ATP release from both surfaces of the uroepithelium, but mucosal release of the nucleotide was 50-fold greater than serosal release (Wang et al., 2005). The

disparity between the site of preferential ATP and adenosine accumulation at the two urothelial surfaces led those authors to hypothesized that hydrolysis of released ATP by ectonucleotidases would play a minor role on adenosine biosynthesis in the rabbit urothelium.

Fine-tuning regulation of extracellular purines in the urothelium is more complex than first hypothesized. Besides adenosine being originated from the breakdown of released adenine nucleotides via the ectonucleotidase pathway, it has been demonstrated that it may negatively modulate stretched-induced ATP release over many minutes by modulating exocytosis in the apical membrane of umbrella cells (Yu et al., 2006). A more acute (within seconds) effect to modulate distension-induced ATP release from the urothelium has been suggested (Dunning-Davies et al., 2013). Distension-induced ATP release was decreased by adenosine (1-10 μM) and enhanced by ADA and the A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), but not by blocking A_2 receptors with 3,7-dimethyl-1-propargyl-xanthine (DMPX). The mechanism by which adenosine reduces urothelial ATP release remains unknown, but it might depend on changes in the transepithelial potential by interference with mechanosensitive Na^+ channels (identified as amiloride-sensitive epithelial Na^+ channels or ENaC), which may ultimately favor Ca^{2+} outflow via Na^+/Ca^{2+} exchange in urothelial cells (Wu et al., 2011). These findings support a dominant role of A_1 receptors in regulating ATP release from the urothelium. This information, which was obtained in rabbit umbrella cells, is in harmony with rat cystometry data showing that luminal application of the A_1 receptor agonist, CCPA, significantly decreased the frequency of the micturition reflex in clear opposition to that observed upon increasing ATP concentration inside the bladder (Kitta et al., 2014). Conflicting results, however exist, as adenosine acting probably via A_1 receptors decreased the threshold pressure needed to stimulate the micturition reflex, thus increasing bladder hyperactivity, in a model of cyclophosphamide-induced cystitis (Prakasam et al., 2012). Whether adenosine exerts diverse effects to control bladder function under pathological conditions versus control situations through coupling to distinctive intracellular signaling pathways, needs clarification.

Whereas adenosine binds to its receptors and impacts organ-protective functions (Cohen & Downey, 2008), chronic elevation of extracellular adenosine is harmful to tissues (Zhou et al., 2010). Hence, the half-life time of extracellular

adenosine is kept short (seconds to minutes, depending on species) through the action of enzymes and transporters. Taking into consideration that adenosine levels at the luminal site of the urothelium are 10-fold below those found in the serosal side, one may hypothesize that the adenosine inactivation mechanisms are particularly active at the luminal surface of the bladder; this has also relevance to keep low urinary adenosine originated upstream the bladder urinary tract (e.g. kidney) (Jackson et al., 2011). In this respect, ADA seems to be most important enzyme to control adenosine levels at the mucosal surface of the bladder, while adenosine kinase and nucleoside transporters seem to play more significant roles at the serosal side of the uroepithelial cell layer (Prakasam et al., 2012). This hypothesis gains further support considering previous studies showing that the basolateral surface of polarized epithelial cells are endowed with equilibrative nucleoside transporters (Loffler et al., 2007).

EFFECTS OF ADENOSINE AT THE DETRUSOR NEUROMUSCULAR JUNCTION

The adenosine content of human bladder smooth muscle is 6.7 times higher than striated muscle and the adenosine/ATP ratio is 1:9 compared with 1:450 for striated muscle (Wedenberg et al., 1994). Several studies have shown that adenosine concentration-dependently relaxes pre-contracted urinary bladder detrusor strips exposed to carbachol, ACh, potassium depolarization, or electrical field stimulation in rats, guinea-pigs (Acevedo et al., 1992; Brown et al., 1979; Burnstock et al., 1978a; King et al., 1997; Nicholls et al., 1992) and humans (Rubinstein et al., 1998). Maximum relaxation by adenosine never exceeded 50% of ACh-induced tension in the isolated human detrusor (Rubinstein et al., 1998). Despite increased ATP-induced contractions have been associated with interstitial cystitis, detrusor overactivity, outlet obstruction, inflammation, neurogenic bladder, spinal cord lesions and aging, there are no reports on different responses to adenosine in these situations.

Characterization of the adenosine receptor subtype responsible for detrusor relaxation has been difficult to establish due to differences in target species and experimental protocols. Based on the rank potency order of P1

agonists to reduce contractile responses caused by ATP or ACh, it seems that an A_2 receptor subtype might be involved (Acevedo et al., 1992; Nicholls et al., 1992). Characterization of the A_2 receptor subtype implicated in this process is hindered by the absence of highly selective and potent ligands for A_{2B} receptors. In the guinea-pig bladder smooth muscle cells adenosine evokes membrane hyperpolarization by activation of K_{ATP} channels involving activation of adenylate cyclase, generation of cyclic AMP and stimulation of protein kinase A (Gopalakrishnan et al., 2002). In this study, the non-selective A_2 receptor agonist, 5'-*N*-ethylcarboxamidoadenosine (NECA), was equipotent to the selective A_{2A} receptor agonist, CGS21680C, and these were more potent than A_1 and A_3 selective agonists, 2-choloro-N^6-cylcopentyladenosine (CCPA) and 2-chloro-N^6-(3-iodobenzyl) adenosine-5'-N-methylcarboxamide (2-Cl-IBMECA), respectively. Moreover, the selective A_{2A} receptor antagonist, ZM241385, inhibited the changes in membrane potential evoked by adenosine and NECA on detrusor smooth muscle cells. However, there are conflicting data with the use of the A_{2A} receptor agonist CGS21680C as it relaxed the guinea-pig detrusor strips pre-contracted with KCl (Gopalakrishnan et al., 2002) but caused only a slight inhibition compared to NECA on carbachol-induced contractions in the urinary bladder of rats (Nicholls et al., 1992) and pigs (Hernandez et al., 1999). This also insinuates a role for low affinity A_{2B} receptors in this process. In fact, the A_{2B} selective antagonist, PSB 1115, blocked the relaxant effect of adenosine when it was applied in high concentrations (Aronsson et al., 2010). Thus, so far there is no clear cut evidence to decide which A_2 receptor subtype predominantly participates in adenosine relaxation of the detrusor muscle (question mark in Figure 5).

Other authors claimed that the A_1 receptor may also partially operate adenosine-induced relaxation of the rat urinary bladder (acting at the detrusor and urothelium), since the selective A_1 receptor antagonist DPCPX, but not the A_{2A} receptor antagonist, SCH58261, blocked the effect of the nucleoside (Vesela et al., 2011). Conversely, findings obtained under different experimental conditions showed that the A_1 receptor may be responsible for contraction of isolated detrusor cells from cats by acting via pertussis toxin-sensitive G_{i3} protein, phospholipase C-β_3 and the release of intracellular Ca^{2+} (Yang et al., 2000). Controversy regarding the effects of adenosine in the urinary bladder detrusor

increases given that data demonstrate that blockage of A_3 receptors may potentiate adenosine relaxation, suggesting that stimulation of A_3 receptors induces detrusor contraction in the rat (Vesela et al., 2011).

Besides direct actions of adenosine on P1 receptors located on detrusor smooth muscle fibers, the nucleoside and its analogues may act indirectly by inhibiting nerve-evoked contractions; this was evidenced in rats (Acevedo et al., 1992; Burnstock et al., 1978a; King et al., 1997; Parija et al., 1991), guinea-pigs (Burnstock et al., 1978a), cats (Yang et al., 2000), and humans (Husted et al., 1983). Interestingly, inhibition of evoked transmitters release by adenosine is much more pronounced than the effect of the nucleoside on pre-contracted human detrusor smooth muscle (Figure 5).

The rank potency order of adenosine analogues to reduce nerve-evoked contractions is clearly different from that required to reduce ATP- and/or carbachol-induced contractions. Being A_1 receptor agonists, like N^6-(R-2-phenylisopropyl)adenosine (R-PIA) and N^6-cyclohexyladenosine (CHA), the most potent ligands on electrically-evoked detrusor contractions, whereas NECA was the most potent agonist on detrusor contractions caused by ATP or carbachol (Acevedo et al., 1992; Nicholls et al., 1992). These findings are consistent with idea that adenosine inhibition of nerve-evoked detrusor contractions is related to the activation of pre-synaptic A_1 receptors (Williams, 1987).

Adenosine also seems to have an important role in NANC, probably nitrergic, inhibition of the bladder neck. ATP-induced relaxation of this tissue seems to be mediated by metabotropic $P2Y_1$ and A_{2A} receptors after its breakdown to ADP and adenosine, respectively (Hernandez et al., 2009).

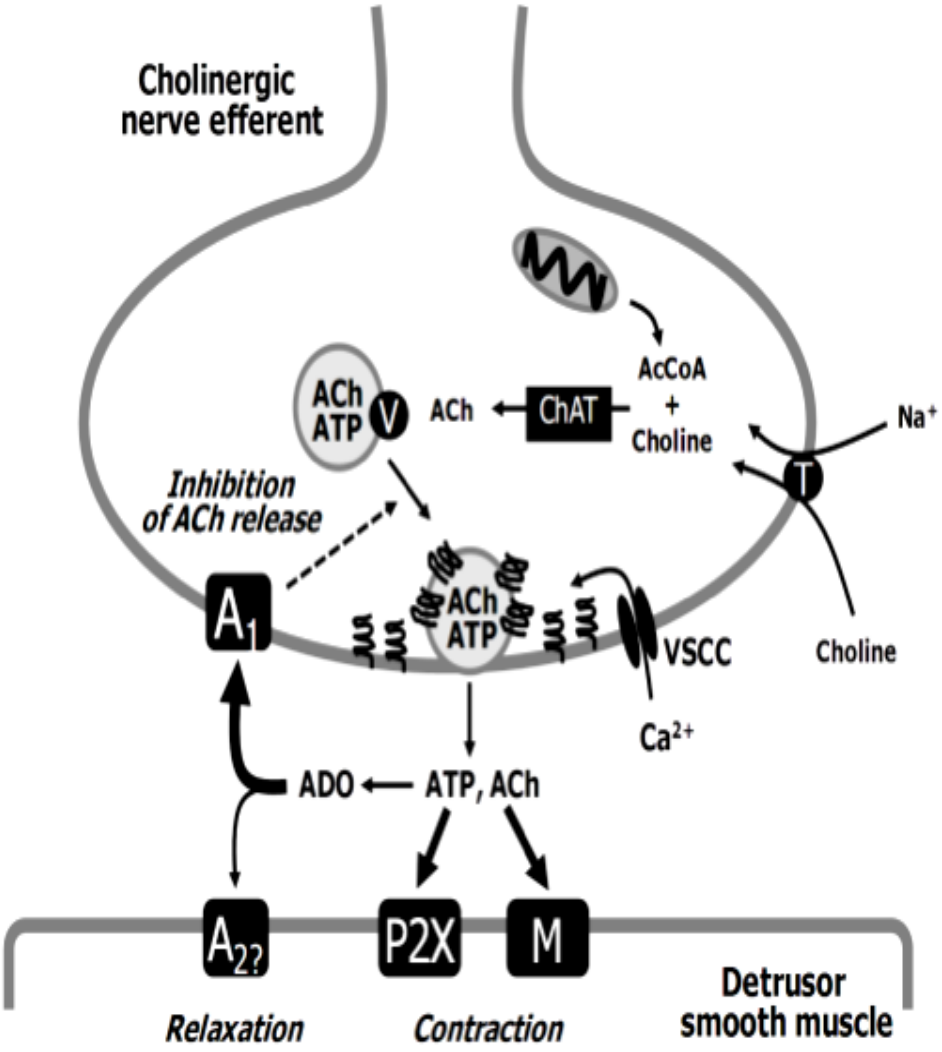


Figure 5. Schematic representation of adenosine effects on the detrusor neuromuscular transmission. Inhibition of nerve-evoked contractions by adenosine in the human detrusor is due to a preferential activation of A_1 receptors on cholinergic nerve efferents, while the nucleoside produces less potent relaxation by directly targeting receptors on smooth muscle fibers. AcCoA represents acetyl coenzyme A, and ChAT, choline acetyl transferase.

EFFECTS OF ADENOSINE IN THE CONTROL OF MICTURITION REFLEX

Adenosine receptors are broadly distributed in the central nervous system being A_1 and A_{2A} receptors the most expressed subtypes (Dixon et al., 1996). The A_1 receptor is widely distributed in the brain, spinal cord and peripheral nervous system (Fredholm et al., 2001), whereas the A_{2A} receptor is predominantly expressed in basal ganglia and in presynaptic terminals of sensory afferents (Dunwiddie & Masino, 2001; Kaelin-Lang et al., 1998).

Most of the evidence for the modulatory role of adenosine on the micturition reflex, both in the periphery and in the central nervous system, comes from cystometry experiments performed in the rat. It has been reported that both R-PIA and NECA adenosine analogues administered intrathecaly delayed the voiding reflex (Sosnowski & Yaksh, 1990). More recently, it was found that activation of A_1 receptors or inhibition A_{2A} receptors in the brain or in the spinal cord cause an inhibitory effect on the micturition reflex (Kitta et al., 2014). These authors demonstrated that amplification of the inhibitory A_1-receptor-mediated effect was observed in the brain of rats with bladder overactivity caused by intravesical instillation of acetic acid, whereas the A_{2A} receptor tonus increased in the spinal cord. A peripheral inhibitory action was observed in this model when A_1 agonists were applied into the lumen of the urinary bladder. Interestingly, A_1 and A_{2A} receptor mediated effects control the micturition reflex acting predominantly on the afferent arm of the reflex, without significant changes in the voiding pressure.

ROLE OF ADENOSINE ON LOWER URINARY TRACT SYMPTOMS

The physiological effect of adenosine is now considered a new direction in halting the progression of damage in several organ systems. During the last decades a huge amount of evidences implicate the purinergic system in the pathogenesis of urinary bladder syndromes (Burnstock, 2014), however not much is known on the role of adenosine in bladder dysfunctions. Nevertheless, a study in a rat model of Parkinson disease suggests that bladder overactivity in this condition was, at least in part, caused by increased activity of A_{2A} receptors in the brain (Kitta et al., 2012). This is not surprising given that these receptors are selectively co-localized and negatively interplay with dopamine D_2 receptors on GABAergic output neurons of the striatopallidal pathway (Mori & Shindou, 2003).

There are also studies suggesting the involvement of P1 receptors in the regulation of the inflammatory response (Hasko et al., 2008; Sitkovsky et al., 2004). In rats with cyclophosphamide induced cystitis, the A_1 receptor activation mediates important pro-inflammatory responses (Aronsson et al., 2010) and paradoxical voiding effects (Prakasam et al., 2012, see above). In fact, blockade

of A_1 receptors with DPCPX normalized bladder contractile responses and prevented inflammatory bladder wall changes induced by cyclophosphamide. The A_1 receptor-mediated relaxation is perhaps replaced by A_{2B} receptors function, while A_3 receptors mediate contraction (Vesela et al., 2011). On the other hand, adenosine activation of A_{2A} has been shown to provide potent anti-inflammatory effects and A_{2A} receptor are highly expressed in immune cells such as monocyte/macrophages, neutrophils and T cells (see e.g. (Sitkovsky et al., 2004). In human urothelial cells infected with *Escherichia coli,* the expression of A_1 and A_{2B} mRNA was decreased and the expression of A_{2A} was increased suggesting that adenosine may regulate inflammatory reactions in response to infection via A_{2A} receptors (Save et al., 2009).

In the quest for new treatment options for OAB, modulation of endogenous adenosine levels and/or P1 receptors activity stands as an interesting prospect.

CHAPTER 2
OUTLINE AND AIMS

“I keep six honest serving-men,
They taught me all I knew;
Their names are What and Why and When
and How and Where and Who.”

Rudyard Kipling

The main objective of this thesis was to investigate changes in the purinergic signalling mechanisms in patients with bladder dysfunctions aiming at unveiling novel targets to diagnose and therapeutically manage these conditions. We approached this task by looking initially to detrusor changes induced by BOO and afterwards looking at changes in the urothelium and ATP concentrations in the urine. Therefore, we could divide this work in two parts: one studying the efferent branch of the micturition reflex by looking at detrusor samples from BPH patients and controls; and a second part where we look at the afferent branch, by studying bladder urothelium and urinary ATP concentrations from patients with LUTD and controls.

On the efferent side of the micturition reflex there are significant gaps on our knowledge on how the purinergic component of atropine-resistant contractions realy work, what are the actual changes on ACh release in the diseased bladder and how purines interact with ACh release. For that matter on Section 3.1 we aimed to fill these gaps by studying smooth muscle contraction, ACh release, purine metabolism and immunolocalization of purinoceptors in detrusor samples from patients with BOO due to BPH and control samples collected from cadaveric organ donors.

On the afferent side, although there is plenty of information on ATP release from nerves and from the urothelium, the assessment of ATP concentrations in urine has not been consistently studied. Consequently, on Section 3.2 we looked at urinary ATP concentration on patients with bladder dysfunctions and assessed the viability of ATP as a non-invasive biomarker of bladder dysfunctions. With that purpose we evaluated urine samples from patients with OAB, BOO and controls.

More specific aims are described in each article.

CHAPTER 3
RESULTS / PUBLICATIONS

3.1 EFFECTS OF PURINES ON ACETYLCHOLINE RELEASE IN THE DETRUSOR

"Oh tell me Lord how could it be,
That though our cells make ATP,
It's not all used for energy,
But sometimes is secreted free.
It puzzles you, it puzzles me,
While Geoffrey Burnstock smiles with glee
At the many roles of ATP."

Samuel C. Silverstein

ARTICLE 1

IMPAIRMENT OF ATP HYDROLYSIS DECREASES ADENOSINE A1 RECEPTOR TONUS FAVORING CHOLINERGIC NERVE HYPERACTIVITY IN THE OBSTRUCTED HUMAN URINARY BLADDER

Miguel Silva-Ramos[1,2,3#], Isabel Silva[1,2#], Miguel Faria[1,2], Maria Teresa Magalhães-Cardoso[1,2], Joana Correia[1,2], Fátima Ferreirinha[1,2] and P. Correia-de-Sá[1,2]

[1]Laboratório de Farmacologia e Neurobiologia, [2]Center for Drug Discovery and Innovative Medicines (MedInUP), Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto, and [3]Serviço de Urologia, Centro Hospitalar do Porto (CHP), Porto, Portugal.

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[#]MSR and IS contributed equally to this work

Author contribution:
Miguel Silva-Ramos: patients recruitment and selection, acquisition, analysis and interpretation of experimental data, statistical analysis, drafting of the manuscript.
Isabel Silva: tissue processing/preservation and "in vitro" experiments, analysis and interpretation of experimental data, statistical analysis
Miguel Faria: myographic recordings, analysis and interpretation of data, statistical analysis.
Maria Teresa Magalhaes-Cardoso: HPLC analysis and data interpretation, statistical analysis.
Joana Correia: immunohistochemistry experiments, analysis and data interpretation.
Fátima Ferreirinha: immunofluorescence staining and confocal microscopy observation,, analysis and interpretation of imaging data.
Paulo Correia-de-Sá: conception and design of the study, project supervision, analysis and interpretation of experimental data, statistical analysis, revision of the manuscript, author for correspondence.

ABSTRACT

This study was designed to investigate whether reduced adenosine formation linked to deficits in extracellular ATP hydrolysis by NTPDases contributes to detrusor neuromodulatory changes associated with bladder outlet obstruction in men with BPH. The kinetics of ATP catabolism and adenosine formation, as well as the role of P1 receptor agonists on muscle tension and nerve-evoked [^{3}H]ACh release were evaluated in mucosal-denuded detrusor strips from BPH patients (n=31) and control organ donors (n=23). The neurogenic release of ATP and [^{3}H]ACh was higher (P<0.05) in detrusor strips from BPH patients. The extracellular hydrolysis of ATP and, subsequent, adenosine formation was slower (t½ 73 vs 36 min, P<0.05) in BPH detrusor strips. The A_1 receptor-mediated inhibition of evoked [^{3}H]ACh release by adenosine (100 μM), NECA (1 μM) and R-PIA (0.3 μM) was enhanced in BPH bladders. Relaxation of detrusor contractions induced by acetylcholine required 30-fold higher concentrations of adenosine. Despite VAChT-positive cholinergic nerves exhibiting higher A_1 immunoreactivity in BPH bladders the endogenous adenosine tonus revealed by adenosine deaminase is missing. Restoration of A_1 inhibition was achieved by favoring (1) ATP hydrolysis with apyrase (2 U.mL^{-1}) or (2) extracellular adenosine accumulation with dipyridamole or EHNA, as these drugs inhibit adenosine uptake and deamination, respectively. In conclusion, reduced ATP hydrolysis leads to deficient adenosine formation and A_1 receptor-mediated inhibition of cholinergic nerve activity in the obstructed human bladder. Thus, we propose that pharmacological manipulation of endogenous adenosine levels and/or A_1 receptor activation might be useful to control bladder overactivity in BPH patients.

INTRODUCTION

Detrusor dysfunction associated with bladder outlet obstruction has long been considered a key factor in the mechanism through which BPH causes persistent LUTS (reviewed in Mirone et al., 2007). Although there is a lack of information regarding the way obstruction may cause bladder dysfunction, morphologic and functional abnormalities of the bladder detrusor are frequent in patients affected with BPH. Most of these changes can be regarded as a consequence of mechanical stress and hypoxic changes caused by BOO at the molecular level in the epithelium, smooth muscle fibres, extracellular matrix, and neuronal network of the urinary bladder (Mirone et al., 2007).

Although it is generally accepted that ACh acting on smooth muscle muscarinic receptors is the primary effector controlling bladder emptying, neural stimulation of the bladder is only partially inhibited by atropine in many species, including humans (Cowan & Daniel, 1983; Dean & Downie, 1978). In keeping with the purinergic hypothesis proposed by Burnstock, it is now accepted that ATP is responsible for the atropine-resistant component of parasympathetic contraction of the detrusor (Burnstock, 2011; Levin et al., 1986). While the purinergic component of detrusor contraction via P2 purinoceptors activation is lacking in healthy humans, it may increase with age (Yoshida et al., 2001) and is responsible for up to 40% of nerve-evoked bladder contractions in pathological conditions, including hypertrophic unstable bladder, overactive detrusor, neurogenic bladder and interstitial cystitis (reviewed in Ruggieri & Braverman, 2006).

In patients with idiopathic detrusor instability, appearance of atropine-resistant P2 purinergic tone is related to decreased ecto-NTPDase activity, thus limiting the breakdown of ATP released from bladder nerves or the urothelium (Harvey et al., 2002). Nonetheless, decreased activity of ecto-NTPDases may also have profound implications for endogenous adenosine formation and P1 receptors activation. Surprisingly this mechanism has never been explored in the human bladder despite the fact that the adenosine content of human bladder smooth muscle is 6.7 times higher than in the striated muscle and the adenosine/ATP ratio is 1:9 compared with 1:450 for skeletal muscle (Wedenberg et al., 1994).

Previous studies have shown that adenosine partially relaxes pre-contracted urinary bladder detrusor strips exposed to carbachol, ACh, or potassium depolarization in rats, guinea-pigs (Acevedo et al., 1992; Brown et al., 1979; Burnstock et al., 1978a; King et al., 1997; Nicholls et al., 1992) and humans (Rubinstein et al., 1998). Besides direct actions of adenosine on P1 receptors located on detrusor smooth muscle fibers, the nucleoside and its analogues may act indirectly by inhibiting nerve-evoked contractions as demonstrated in rats (Acevedo et al., 1992; Burnstock et al., 1978a; King et al., 1997; Parija et al., 1991), guinea-pigs (Burnstock et al., 1978a), cats (Yang et al., 2000) and humans (Husted et al., 1983). All four subtypes of adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) have been identified in the bladder of experimental animals and humans by RT-PCR analysis; results show that A_{2A} and A_{2B} mRNAs are more expressed than A_1, with the A_3 being the least expressed receptor (Dixon et al., 1996; Owen et al., 2012). Discrepancy between the relative abundance of A_1 and A_2 receptor subtypes in the detrusor (Dixon et al., 1996) may be attributed to their preferential localization on tiny nerve terminals and smooth muscle fibers, respectively (Acevedo et al., 1992; Nicholls et al., 1992), which supports previous pharmacological data obtained in rodents. There are, however, no published reports on subtype distribution of adenosine receptors in the human urinary bladder.

Therefore, this study was designed to investigate the kinetics of ATP catabolism and adenosine formation, as well as the role of specific adenosine receptor ligands on electrically-evoked [^{3}H]ACh release and muscle tension in mucosal-denuded detrusor strips, which were collected from men with outflow obstruction due to BPH (patients) and control organ donors. Because RT-PCR results do not necessarily correlate with the presence of functional adenosine receptors on the cell surface, we evaluated the expression of adenosine receptor subtypes in the human detrusor by immunofluorescence confocal microscopy.

PATIENTS AND METHODS

Human detrusor samples.

Samples of human detrusor were collected from the bladder dome of thirty one male patients (62±6 years of age) with bladder outlet obstruction due to BPH submitted to transvesical prostatectomy and from twenty three male organ

donors (56±4 years of age) at the time of harvesting organs for transplantation. BOO and prostate enlargement were confirmed by uroflowmetry and ultrasonography, respectively. Collected samples were immediately placed at 4-6°C in mannitol transplantationsolution at 400 mOsm/kg (M-400) not supplemented with ATP or adenosine (230 mM mannitol, 15 mM KH_2PO_4, 43 mM $K_2HPO_4 \cdot 3H_2O$,15 mM KCl, and 10 mM $NaHCO_3$, pH 7.4) and transported to the laboratory. Experiments were performed within the first 24 h after collection, which corresponds to the tissue viability window. This study and all its procedures were approved by the Ethics Committees of CHP and ICBAS-UP and were authorized by the National Transplantation Committee. All BPH patients signed an informed consent approved by the Ethics Committee of CHP for using the biological material. Regarding deceased organ donation, the legal frame work allows the "Presumed Consent" stating that residents in Portugal are consenting donors unless the individual previously objected during his or her life. The investigation conforms to the principles outlined in *The Code of Ethics of the World Medical Association* (Declaration of Helsinki).

Quantification of ATP and [^{3}H]ACh release.

After dissecting out the mucosa through cleavage at the *lamina propria*, detrusor muscle strips (~1.5mm x 5mm) were mounted in 3-mL capacity vertical perfusion chambers heated at 37°C. The procedures used for labeling the preparations and measuring evoked [^{3}H]ACh release were described previously (Carneiro et al., 2014; Correia-de-Sa et al., 2006; Duarte-Araújo et al., 2004). Briefly, the preparations were superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution (pH 7.4) containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001. After a 30 min equilibration period, cholinergic neurons were loaded with 1 μM [^{3}H]choline (specific activity 2.5 μCi nmol^{-1}) under electrical field stimulation (EFS, 1 Hz-frequency, 0.5 ms pulse width, 40 V) during 40 min. Washout of the preparations was performed for 60 min, by superfusion (15 mL min^{-1}) with Tyrode's solution supplemented with the choline uptake inhibitor, hemicholinium-3 (10 μM). Tritium content was measured by liquid scintillation spectrometry (TriCarb2900TR, Perkin Elmer, Boston, USA) (% counting efficiency: 58±2%) after appropriate background subtraction, using 400-μL bath samples collected automatically

every 3 min with a fraction collector (Gilson, FC203B, France). In control experiments, aliquots of matched samples were immediately freeze-dried in liquid nitrogen and preserved at -80ºC for subsequent ATP quantification by bioluminescence using the luciferin-luciferase ATP kit HS II (Roche Applied Science, Indianapolis, USA) according to manufacturer's instructions. Luminescence was determined using a multi-detection microplate reader (Synergy HT, BioTek Instruments) (Carneiro et al., 2014; Pinheiro et al., 2013; Timóteo et al., 2014) .

[^{3}H]ACh release was evoked by two periods of electrical field stimulation (S_1 and S_2, 200 pulses of 0.5 ms duration delivered at 10 Hz frequency). Therefore, the evoked [^{3}H]ACh release was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period (see *e.g.*(Carneiro et al., 2014; Correia-de-Sá et al., 2006; Duarte-Araújo et al., 2004). Likewise, stimulation-evoked release of ATP was calculated by subtracting the basal release, measured in the sample collected before stimulation, from the total release of the nucleotide determined after stimulus application (Carneiro et al., 2014; Pinheiro et al., 2013; Timóteo et al., 2014).

Myographic recordings

Detrusor muscle strips without the mucosa were mounted in 10-mL capacity perfusion chambers connected to isometric force transducers. The changes in tension were recorded continuously with a PowerLab data acquisition system (Chart 5, v.4.2; AD Instruments, USA). Tissues were preloaded with 5 mN of tension and allowed to equilibrate for 90 min in Tyrode's solution, at 37ºC. To evaluate the inhibitory role of adenosine (0.03-3 mM) and its enzymatically stable analogue, 5'-(N-ethylcarboxamide) adenosine (NECA, 0.3-300 μM), on detrusor strips contractions induced by ACh (10 μM), these drugs were added to the incubation media 6 min before ACh which contacted with the preparations for 2 min before washout of all drugs. Under these experimental conditions, exogenous ACh (0.1-1000 μM) concentration-dependently increased myogenic contractions of detrusor strips from control individuals and BPH patients with a similar potency (EC_{50} 3 μM); a higher (P<0.05) maximal contraction amplitude was achieved in control preparations (4.74±0.46 mN/mg of wet weight, n=8) than in obstructed human bladders (2.64±0.27 mN/mg of wet weight, n=14). ACh,

applied in the low micromolar concentration range close to the EC_{50} value, has been instrumental to evoke sustained myogenic contractions to investigate the effects of detrusor smooth muscle relaxants (see *e.*g. Parija et al., 1991; Husted et al., 1983).

Kinetic of the extracellular catabolism of adenine nucleotides by HPLC

For the kinetic experiments of the extracellular catabolism of adenine nucleotides, detrusor strips without the mucosa were mounted in a 2-mL organ bath. All experiments were performed at 37°C. Preparations were superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution. After equilibrium, the preparations were incubated with 30 µM of ATP or AMP (zero time). Samples of 75 µl were collected from the organ bath at different times up to 45 min for HPLC (with UV detection) analysis (LaChrome Elite, Merck, Germany) of the variation of substrate disappearance and product formation (Correia-de-Sá et al., 2006; Duarte-Araújo et al., 2004; Vieira et al., 2014). The stoichiometry of ATP and AMP conversion into their metabolites was kept unaltered (30 µM). Considering that the curvilinear decrease of the initial substrate with time is characteristic of first-order kinetics, the half-degradation time was estimated frompolynomial fitting of linear semi-logarithmic progress curves of the catabolism of adenine nucleotides for each separate experiment (see *e.g.* Duarte-Araújo et al., 2004).

Immunofluorescence staining and confocal microscopy observation

Detrusor strips without the mucosa were stretched in all directions and pinned onto a Petri dish coated with Sylgard®. The strips were then fixed in PLP solution (paraformaldehyde 2%, lysine 0.075 M, sodium phosphate 0.037 M, sodium periodate 0.01 M) for 16 h at 4ºC. Sixteen-micron sections were incubated with selected primary antibodies (Table 1) diluted in an incubation buffer (foetal bovine serum 5%, serum albumin 1%, Triton X-100 0.3% in PBS), at 4 ºC, for 16 h. For double immunostaining, antibodies were combined before application to tissue samples. After washing away unbound primary antibody, the sections were incubated with secondary antibodies in the dark for 2 hours, at room temperature. Negative controls were carried out by replacing the primary antibodies with non-immune serum; cross-reactivity of the secondary antibodies was tested in control experiments in which primary antibodies were omitted.

Specificity of primary antibodies used to target human P1 receptors were all previously validated in heterologous expression systems according to references provided in manufacturers' websites. Finally, tissue samples were mounted on optical-quality glass slides using VectaShield as antifade mounting media (VectorLabs) and stored in the dark at 4 ºC. Observations were performed and analyzed with a laser-scanning confocal microscope (Olympus FluoView, FV1000, Tokyo, Japan). During documentation of detrusor sections from control and BPH patients, settings on the confocal microscope were kept unaltered to minimize bias.

Table 1 – Primary and secondary antibodies used to label human detrusor strips

Primary Antibodies	Code	Host	Dilution	Source
Anti-VAChT	ab69000	Goat (gt)	1:75	Abcam
Anti-A_1 receptor	ab75177	Rabbit (rb)	1:250	Abcam
Anti-A_{2A} receptor	sc-13937	Rabbit (rb)	1:75	Santa Cruz
Anti-A_{2B} receptor	ab1589P	Rabbit (rb)	1:75	Abcam
Anti-A_3 receptor	sc-7508	Goat (gt)	1:50	Santa Cruz
Secondary Antibodies	**Code**	**Host**	**Dilution**	**Source**
Alexa Fluor 488 anti-rb	A-21206	Donkey	1:1500	Molecular probes
Alexa Fluor 568 anti-gt	A-11057	Donkey	1:1500	Molecular probes

Drugs and Solutions

Acetylcholine, adenosine deaminase (ADA, type VI, 1803 $U.mL^{-1}$, EC 3.5.4.4), ATP, ADP, AMP, adenosine, apyrase (from potato, EC 3.6.1.5), EHNA, inosine, hemicholinium-3, R-PIA, NECA, DPCPX, choline chloride, paraformaldehyde (prills), lysine, sodium periodate, anhydrous glycerol, fetal bovine (Sigma, St Louis, MO, USA); dipyridamole (Boehringer Ingelheim, Germany); [methyl-^{3}H] Choline chloride (etanol solution, 80.6 $Ci.mmol^{-1}$)

(PerkinElmer, Boston, USA); serum albumin, Triton X-100 (Merck,Darmstadt, Germany). EHNA was dissolved in a 5 mM stock solution in ethanol. DPCPX was dissolved in a 5 mM stock solution in 99% dimethylsulphoxide + 1% NaOH 1M ($v.v^{-1}$). ZM 241385 and R-PIA were dissolved in 5 and 50 mM stock solutions in DMSO, respectively. All stock solutions were stored as frozen aliquots at -20ºC. Dilutions of these stock solutions were made daily and appropriate solvent controls were done.

Presentation of data and statistical analysis

Results are expressed as mean ± SD, with *n* indicating the number of individuals used for a particular set of experiments. Only one experimental procedure (*e.g.* agonist in the absence and in the presence of the antagonist) was performed per individual. Statistical analysis of data was carried out using Graph Pad Prism 6.04 for Windows software (La Jolla, USA). Paired and unpaired Student's *t*-test with Welch's correction was used for statistical analysis when parametric data was considered. One-way analysis of variance (ANOVA) followed by the Holm-Sidak correction was used for multiple comparisons. $P<0.05$ (two-tailed) values were considered statistically significant.

RESULTS

Downregulation of ecto-NTPDase1/CD39 and ecto-5'-nucleotidase/CD73 lead to deficient adenosine formation in the detrusor of BPH patients

Figure 6 illustrates the kinetics of the extracellular catabolism of ATP (30 μM) and, subsequent, formation of ADP and AMP in mucosal-denuded detrusor strips from control individuals and BPH patients. Extracellular ATP (30 μM) was hydrolyzed with a half-degradation time of 36±5 min (n=6) in control individuals (Figure 6A), but the catabolism of the nucleotide was significantly ($P<0.05$) slower ($t½$ = 73±16 min, n=5) in detrusor strips from BPH patients (Figure 6B) In both groups, the amount of AMP generated in the extracellular milieu was higher ($P<0.05$) than that of ADP at all time points considered after ATP (30 μM) application (Figure 6, panels C and D).

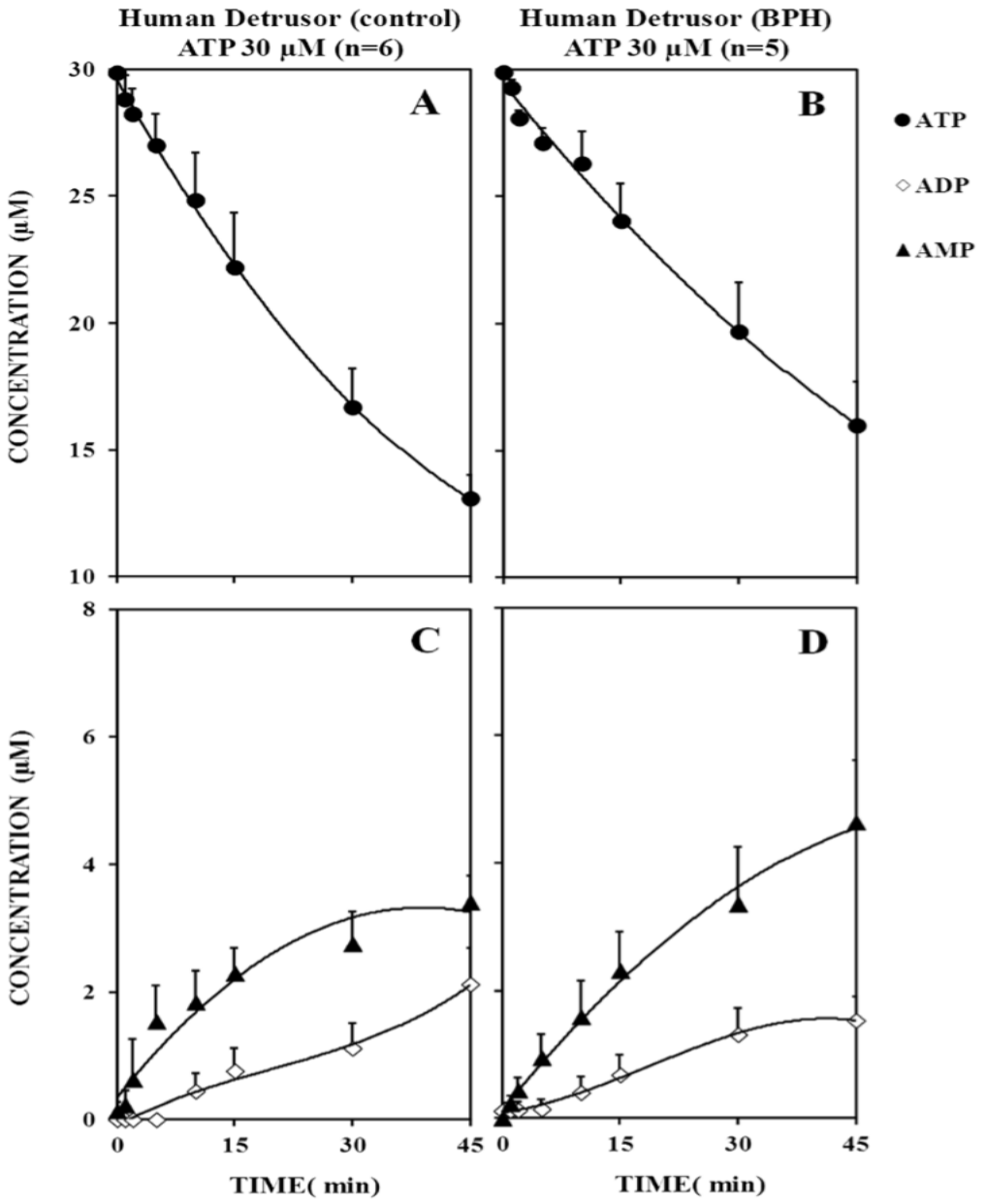

Figure 6 – The extracellular catabolism of ATP is slower in mucosal-denuded detrusor strips from patients with outflow obstruction due to benign prostatic hyperplasia (BPH) as compared to control organ donors (Control). ATP (30 µM) was added at zero time to the incubation medium. Samples were collected from the incubation fluid at the indicated times on the abscissa and analysed by HPLC with UV detection to quantify ATP (filled circles) and its metabolites, ADP (open lozenges) and AMP (filled triangles). Average results obtained from six control individuals (panels A and C) and five BPH patients (panels B and D); the vertical bars represent SD and are shown when they exceed the symbols in size.

This pattern suggests a dominant involvement of ecto-NTPDase1/CD39 (also called ATP diphosphohydrolase or apyrase, EC 3.6.1.5) converting extracellular ATP directly into AMP, removing two phosphate at a time with almost

no release of the intermediate ADP. As a matter of fact, the activity of ecto-NTPDase1/CD39 calculated 5-min after ATP (30 μM) application by the ratio of [AMP]:[ATP+ADP] per min decreased (P<0.01) from 11.40±0.85 x10^{-3} (n=6) in control subjects to 6.94±0.53 x10^{-3} (n=5) in BPH patients, while up to this time point no ADP could be detected in incubation media (Figure 6, panels C and D).

Figure 7 shows that extracellular AMP (30 μM) is subsequently dephosphorylated into adenosine, inosine and hypoxanthine in the human detrusor. Interestingly, hydrolysis of AMP (30 μM) to adenosine and inorganic phosphate by ecto-5'-nucleotidase/CD73 (EC 3.1.3.5) was significantly (P<0.05) diminished in bladder samples from BPH patients ($t½$ = 79±7 min, n=5; Figure 7B) compared to control individuals ($t½$ = 45±4 min, n=5; Figure 7A). Impairment of the extracellular AMP (30 μM) catabolism in the detrusor of BPH patients led to a significant (P<0.01) decrease in adenosine formation (Figure 7D) compared to control individuals (Figure 7C); this difference reached a maximum of 4.14±0.58 μM (n=5) at 10-min incubation time which diminished thereafter. The decrease in the rate of adenosine formation from extracellular AMP was also accompanied by a deficient generation of inosine resulting from the irreversible deamination of adenosine catalyzed by ecto-adenosine deaminase (ecto-ADA, also known as adenosine aminohydrolase, EC 3.5.4.4) (Figure 7, panels C and D). Inosine was then deribosylated by purine nucleoside phosphorylase (EC 2.4.2.1) leading to small amounts of hypoxanthine.

All together these results indicate that adenosine formation from the extracellular catabolism of adenine nucleotides is impaired in the detrusor of BPH patients due to deficient activity of both ecto-NTPDase1/CD39 and ecto-5'-nucleotidase/CD73.

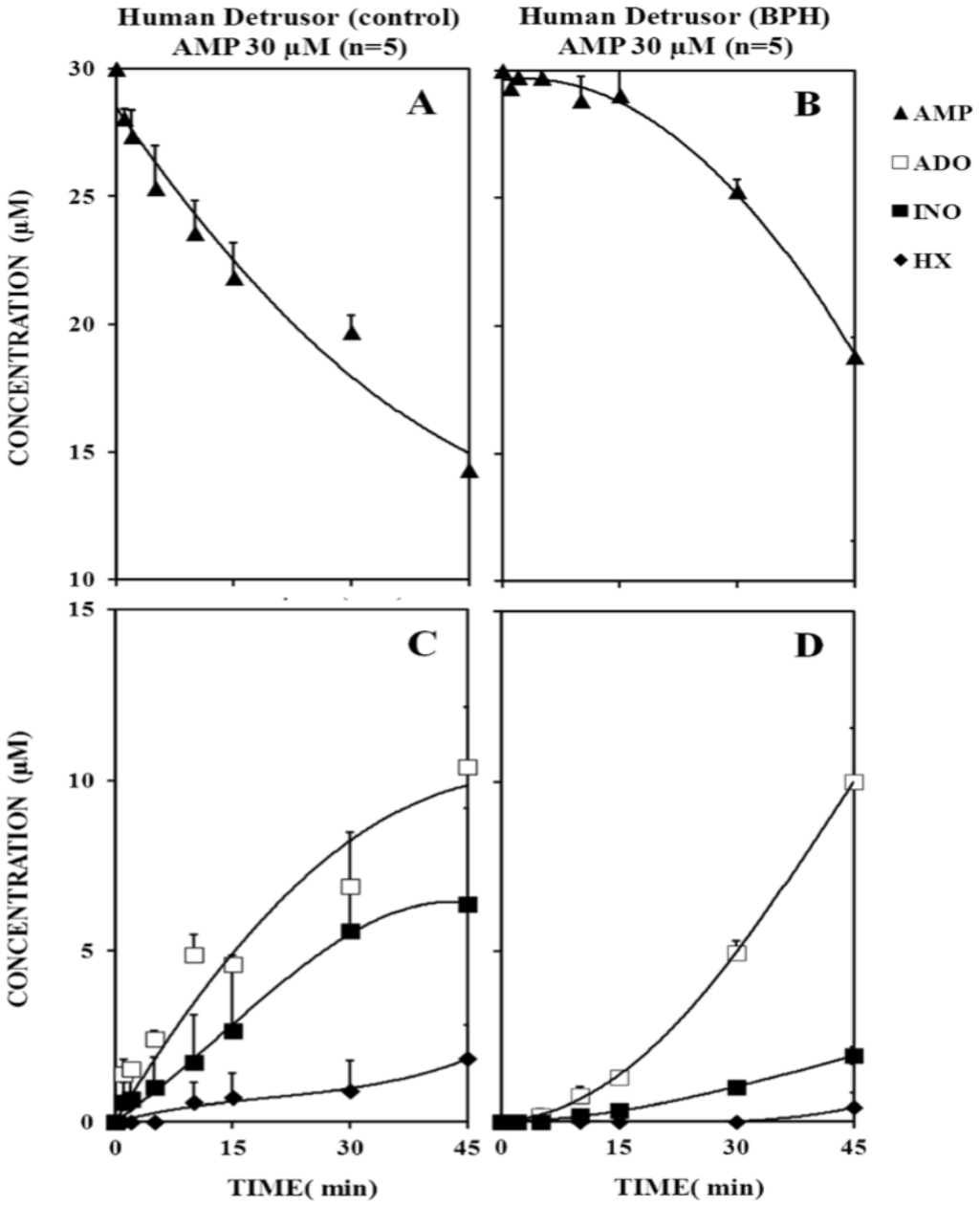

Figure 7 – The kinetics of extracellular AMP dephosphorylation is slower in mucosal-denuded detrusor strips from patients with outflow obstruction due to benign prostatic hyperplasia (BPH) as compared to control organ donors (Control). AMP (30 µM) was added at zero time to the incubation medium. Samples were collected from the incubation fluid at the indicated times on the abscissa and analysed by HPLC with UV detection to quantify AMP (filled triangles) and its metabolites, adenosine (ADO, open squares), inosine (INO, filled squares) and hypoxanthine (HX, filled lozenges). Average results obtained from five individuals of each group, control (panels A and C) and BPH (panels B and D); the vertical bars represent SD and are shown when they exceed the symbols in size

Exogenous NTPDase1/CD39 (apyrase) re-adjusts cholinergic nerve hyperactivity to control levels in the detrusor of BPH patients

Electrical field stimulation of detrusor strips increases the outflow of [^{3}H]ACh above the basal level (Figure 8). Prevention of the evoked tritium outflow in the absence of calcium ($Ca^{2+}\varnothing$ + EGTA, 1 mM) and in the presence of tetrodotoxin (1 µM) indicates that [^{3}H]ACh release results from vesicle exocytosis of depolarized nerve terminals (data not shown). The amount of [^{3}H]ACh released from stimulated cholinergic nerves during S_1 was 1.5-fold higher (P<0.001) in the detrusor of BPH patients (27.6±2.7 x 10^3 DPM.g^{-1} of wet tissue weight, n=12) than in control organ donors (11.1±0.2 x 10^3 DPM.g^{-1} of wet tissue weight, n=14). In some of the experiments we measured in parallel the ATP content of collected samples. Electrical stimulation of detrusor strips from BPH patients produced 2.5-fold higher (P<0.001) amounts of ATP in the incubation fluid (4.9±0.4 pM, n=4) than those observed in control individuals (1.4±0.2 pM, n=8). Exogenously added apyrase (2 U.mL^{-1}), the enzyme that catalyses ATP inactivation into AMP bolstering the formation of adenosine, decreased [^{3}H]ACh release from stimulated cholinergic nerves. The inhibitory effect of apyrase (2 U.mL^{-1}) was more evident (P<0.05) in detrusor strips from BPH patients (18±6%, n=4) than from control subjects (2±4%, n=4), *i.e.* in conditions where extracellular ATP accumulation was higher (Figure 8).

Adenosine A_1 receptors located on cholinergic nerve endings play a dominant role to reduce detrusor activity in the human bladder

Despite the observation that adenosine is able to relax pre-contracted detrusor strips from the human bladder (Rubinstein et al., 1998), we found that this is only valid for concentrations of the nucleoside in the millimolar range (Figure 9).

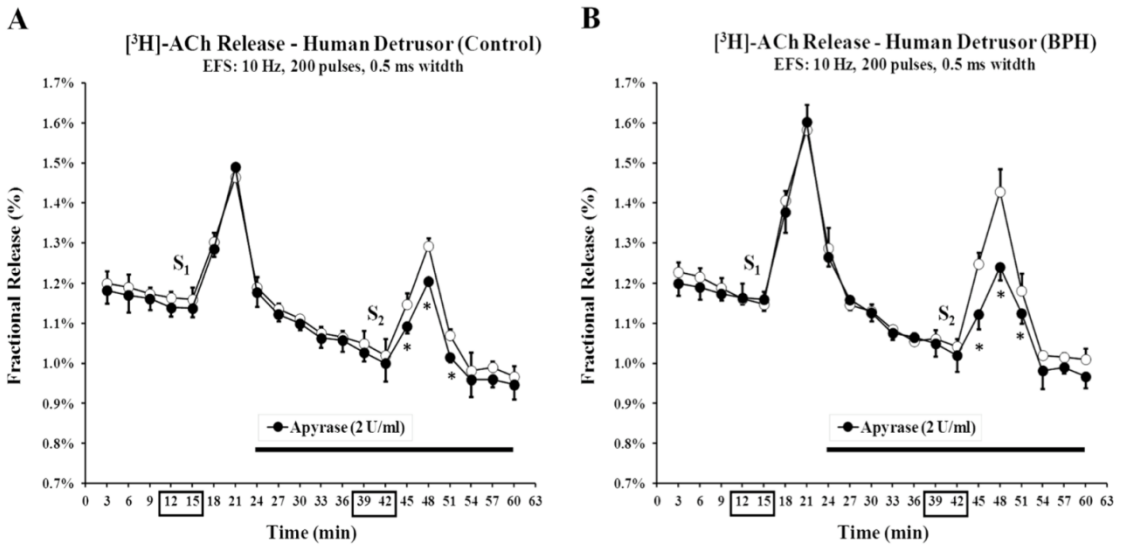

Figure 8 - Effects of apyrase on electrically-evoked [^{3}H]ACh release from urothelium-denuded detrusor strips from control individuals (A) and patients with outflow obstruction due to BPH (B). Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period (Fractional release, %) (see *e.g.* (Duarte-Araújo et al., 2004). Abscissa indicates the times at which samples were collected. [^{3}H]ACh release was elicited by electrical field stimulation (10 Hz, 200 pulses of 0.5 ms duration) twice, starting at 12th (S_1) and 39th (S_2) minutes after the end of washout (zero time). Apyrase (2 U.mL^{-1}, closed circles) was added to the incubation media 15 min before S_2 (horizontal bar). The vertical bars represent SD of four different individuals. *P<0.05 (unpaired Student's *t*-test with Welch's correction) represent significant differences when compared to the situation without apyrase. Note that the spontaneous tritium outflow was not changed in the presence of apyrase.

In our hands, adenosine reduced acetylcholine (10 μM) induced contractions of BPH patient's detrusor only when it was applied in a 3 mM concentration (Figure 9B), but not when the nucleoside was tested in lower (eventually more physiological) amounts (30 and 300 μM). The enzymatically stable adenosine analogue, NECA (0.3-300 μM), which activates preferentially A_2 receptors (IC_{50} ~ 16 nM), was unable to cause relaxation of the pre-contracted human detrusor (Figure 9A and 9B), notwithstanding the fact that A_{2A} and/or A_{2B} subtypes are considered the most expressed receptors in the detrusor smooth muscle (Dixon et al., 1996; Owen et al., 2012). Water solubility limitation precluded the use of millimolar concentrations of NECA.

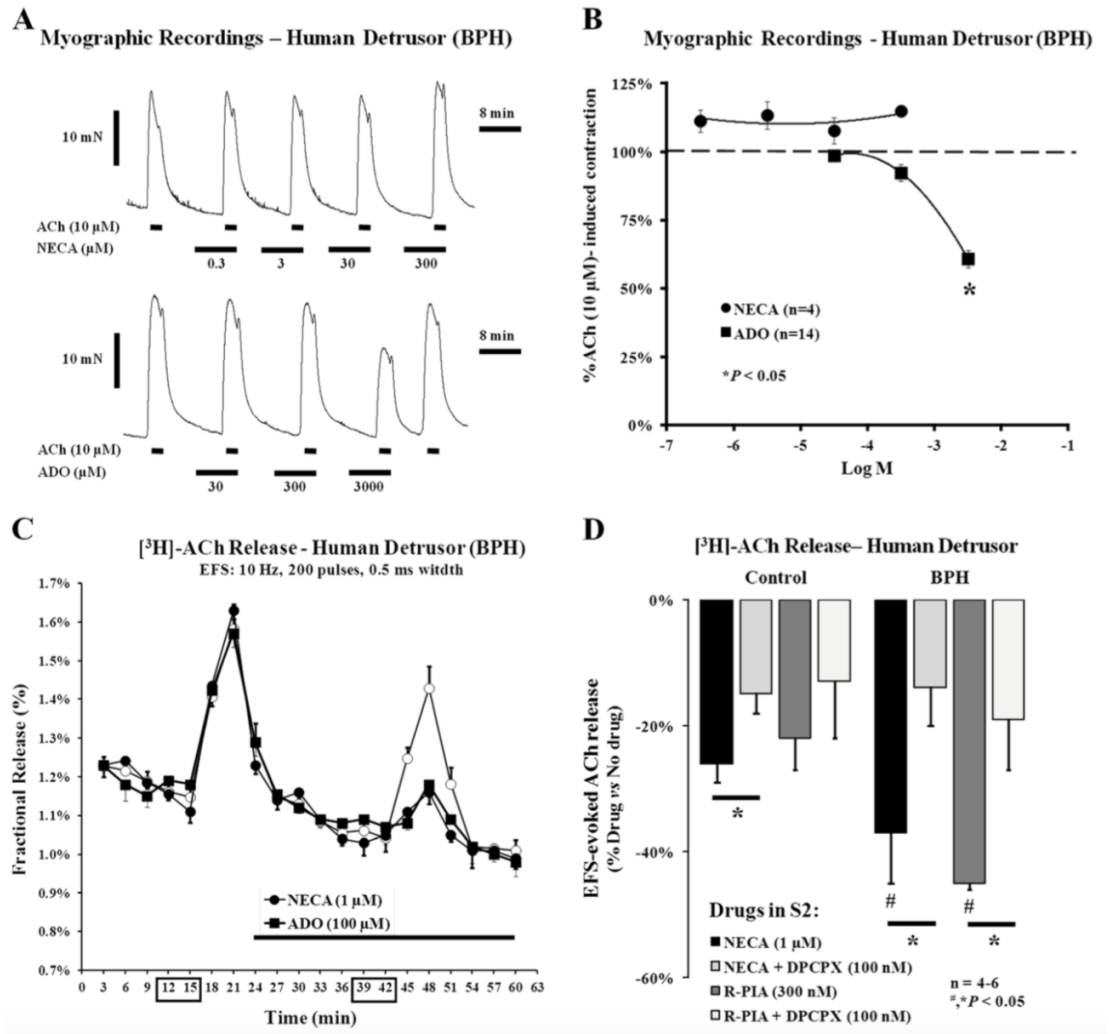

Figure 9 – Adenosine and its stable analogues, NECA and R-PIA, preferentially activates DPCPX-sensitive inhibitory A_1 receptors on cholinergic nerve terminals of the human detrusor and their potency increase in patients with outflow obstruction due to benign prostatic hyperplasia (BPH) as compared to controls. **(A)** Shows myographic recordings of mucosal-denuded detrusor strips from BPH patients. Contractile responses were elicited by ACh (10 μM) applied either in the absence or in the presence of NECA (0.3-300 μM) and adenosine (30-3000 μM), which concentration-response curves are shown in **(B).** The vertical bars represent SD of an *n* number of individuals. **P*<0.05 (unpaired Student's *t*-test with Welch's correction) represent significant differences when compared to the control situation without any drug added (100%, horizontal dashed line). **(C)** Effects of NECA (1 μM) and adenosine (100 μM) on electrically-evoked [^{3}H]ACh release from mucosal-denuded detrusor strips of BPH patients; NECA (1 μM) and adenosine (100 μM) were added to the incubation media 15 min before S_2 (horizontal bar). The vertical bars represent SD of four different individuals. **(D)** Shows the inhibitory effects of NECA (1 μM) and R-PIA (300 nM) on electrically-evoked [^{3}H]ACh release from detrusor strips of organ donors (control) and BPH patients in the absence and in the presence of DPCPX (100 nM); the selective A_1 receptor antagonist was present throughout the assay, including S_1 and S_2. The ordinates are changes in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained without addition of any drug. The data are means ± SD of four to six individuals. $^{\#,*}P$<0.05 (unpaired Student's *t*-test with Welch's correction) represent significant differences when compared to control individuals or to the situation where the adenosine analogue was tested in the absence of DPCPX, respectively.

Figure 9 (panels C and D) also shows that adenosine (100 μM) and its analogues, NECA (1 μM) and R-PIA (0.3 μM, a selective A_1 receptor agonist), inhibit [^{3}H]ACh release from stimulated cholinergic nerves of the human detrusor, when these drugs were used in concentrations unable to cause relaxation of myogenic contractions induced by acetylcholine (10 μM, EC_{50}~3 μM). The inhibitory effects of NECA (1 μM) and R-PIA (0.3 μM) were of higher magnitude in BPH patients than in control individuals (Figure 9D). Likewise, the native compound adenosine (100 μM) diminished nerve-evoked [^{3}H]ACh release by 9±8% (*n*=5, *P*>0.05) and 39±2% (*n*=5, *P*<0.05) in the detrusor of control organ donors and BPH patients, respectively. The selective A_1 receptor antagonist, DPCPX (100 nM), significantly (*P*<0.05) attenuated the inhibitory effects of NECA (1 μM) and R-PIA (0.3 μM) on evoked [^{3}H]ACh release (Figure 9D), thus suggesting that A_1 receptors located on cholinergic nerve endings exert a dominant effect to reduce detrusor hyperactivity in the human bladder.

The immunolocalization studies shown in Figure 10A suggest that A_1 and A_{2A} are the most expressed receptors in the human detrusor, whereas immunoreactivity of A_{2B} and A_3 receptors is less evident. Co-localization experiments show for the first time a differential distribution of the A_1 receptor, which is localized preferentially on vesicular acetylcholine transporter (VAChT)-positive cholinergic nerves (Figure 10B), whereas the A_{2A} receptor is diffusely expressed on smooth muscle fibers of the human detrusor (Figure 10A, panel E). Interestingly, the A_1 receptor immunolabelling becomes more intense in the detrusor of obstructed BPH patients as compared to control individuals (Figure 10A, respectively panels A and C). The relative increase in A_1 receptor immunoreactivity in the detrusor of obstructed BPH patients was not evidenced for any other P1 receptor subtype.

Inhibition of adenosine uptake and/or deamination rehabilitates the A_1 receptor inhibitory tonus in the detrusor of BPH patients

To study the net tonic inhibitory effect of endogenous adenosine on [^{3}H]ACh release from stimulated cholinergic nerves of the human detrusor, we tested the effect of adenosine deaminase (ADA, 0.5 U.mL^{-1}), the enzyme that inactivates adenosine by converting it into inosine (Arch & Newsholme, 1978a).

In contrast to apyrase (2 U.mL^{-1}) that catalyses ATP metabolism into AMP bolstering the formation of adenosine (see also Figure 8), ADA (0.5 U.mL^{-1}) had virtually no effect (P>0.05) on evoked [^{3}H]ACh release from the human detrusor (Figure 11).

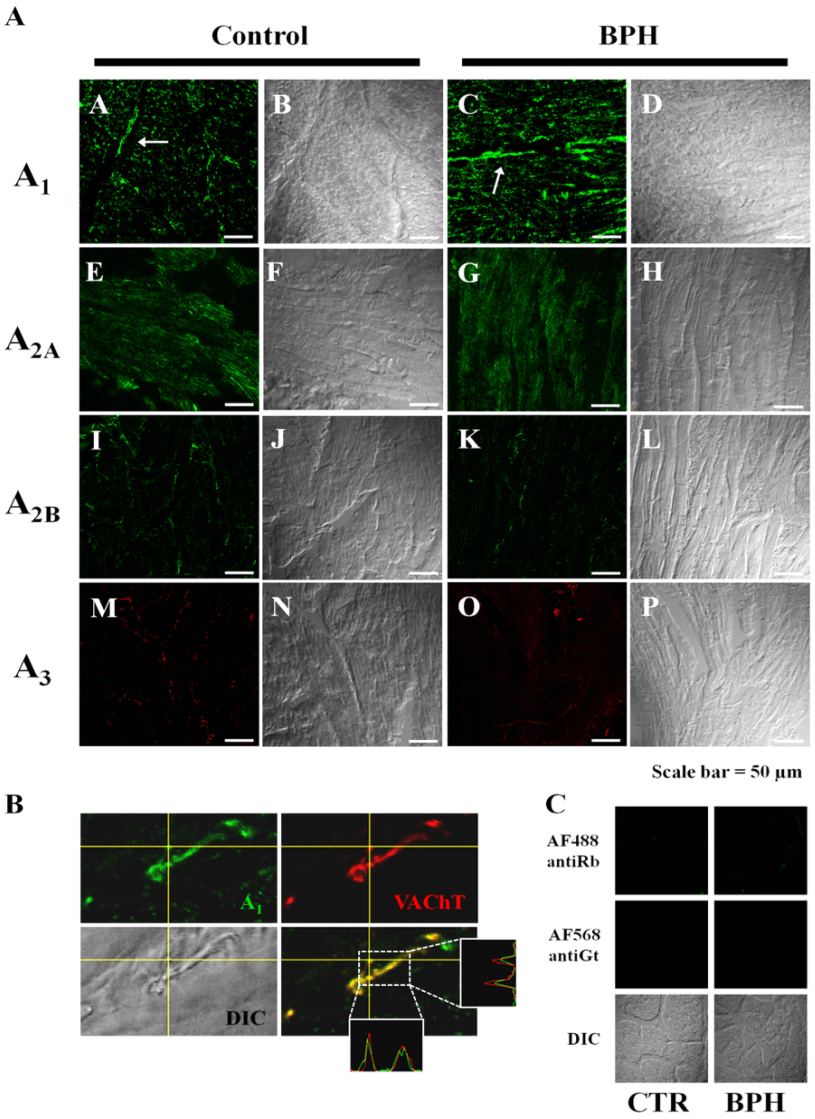

Figure 10 – (A) Confocal micrographs showing the immunoreactivity against A_1, A_{2A}, A_{2B} and A_3 receptors in transverse sections of the detrusor from organ donors (control) and BPH patients. A_1, A_{2A}, A_{2B} and A_3 receptor immunoreactivity is shown in green. Images are representative of five individuals per group, Control and BPH. A_1 and A_{2A} are the most expressed receptors in the human detrusor (panels A-H), whereas immunoreactivity of A_{2B} and A_3 receptors is only vestigial (panels I-P). The A_1 receptor immunolabelling is more evident (arrows) in the detrusor of obstructed BPH patients (panel C) as compared to controls (panel A). Scale bars = 50 µm. (B) Co-localization experiments show that the A_1 receptor is localized with VAChT in cholinergic nerve terminals; yellow staining denotes co-localization.Fluorescence intensity plots drawn from lines crossing regions of interest delineated in the overlay image clearly evidence co-localization of A_1 receptors (green) and VAChT-positive (red) cholinergic nerve terminals. (C) Cross-reactivity of secondary antibodiesin control experiments in which primary antibodies were omitted(see Table 1). Differential interference contrast (DIC) images are also shown for comparison.

Discrepancies between the effects of ADA (0.5 $U.mL^{-1}$) and apyrase (2 $U.mL^{-1}$) prompted us to evaluate whether the A_1-receptor-mediated inhibitory control of [^{3}H]ACh release from stimulated cholinergic nerves could emerge by favoring endogenous adenosine accumulation in the human detrusor with dipyridamole (0.5 μM, a nucleoside uptake blocker) (Griffith & Jarvis, 1996) or EHNA (50 μM, an adenosine deaminase inhibitor) (Agarwal et al., 1977). These drugs reduced [^{3}H]ACh release from stimulated cholinergic nerve terminals. The inhibitory effects of adenosine uptake and deamination blockers were not significantly (P>0.05) different in bladders from control individuals and BPH patients (Figure 11). Pretreatment with the selective A_1 receptor antagonist, DPCPX (100 nM), abolished the inhibitory effects of both dipyridamole (0.5 μM, 9±6%, n=4) and EHNA (50 μM, 7±1%, n=5) on evoked [^{3}H]ACh release from detrusor strips of BPH patients, indicating that their effects are indeed mediated by increases in the extracellular concentration of adenosine leading to activation of membrane-bound A_1 inhibitory receptor.

DISCUSSION

It is widely accepted that purines, in particular ATP, are involved in a number of physiological processes in the lower urinary tract. ATP was shown (i) to be a co-transmitter with ACh in the parasympathetic control of bladder contraction (Burnstock et al., 1972), (ii) to activate sensory nerves during bladder filling conveying both normal and abnormal sensations like urgency and pain (Aizawa et al., 2011; Birder & Andersson, 2013), and (iii) to participate in the central control of bladder reflexes (Rocha et al., 2001). However, not so much is known about the effects of adenosine, the breakdown product of extracellular ATP metabolism. First reports of the effect of adenosine in the lower urinary tract suggested that it reduces the tone and spontaneous activity of the guinea-pig urinary bladder (Burnstock et al., 1978b; Burnstock et al., 1972).

ATP release from both neuronal and non-neuronal cells (e.g. urothelial cells, interstitial cells, fibroblast-like cells, smooth muscle fibers) and its extracellular metabolism into adenosine has been observed in the urinary bladder of rodents (Cusack & Hourani, 1984; Yu et al., 2011; Zhang et al., 1991). Roughly 50% of the extracellular adenosine results from the breakdown of ATP (Smith &

Lu, 1991) which is currently regarded to be the most important route of generating extracellular adenosine (Zhang & Xia, 2012).

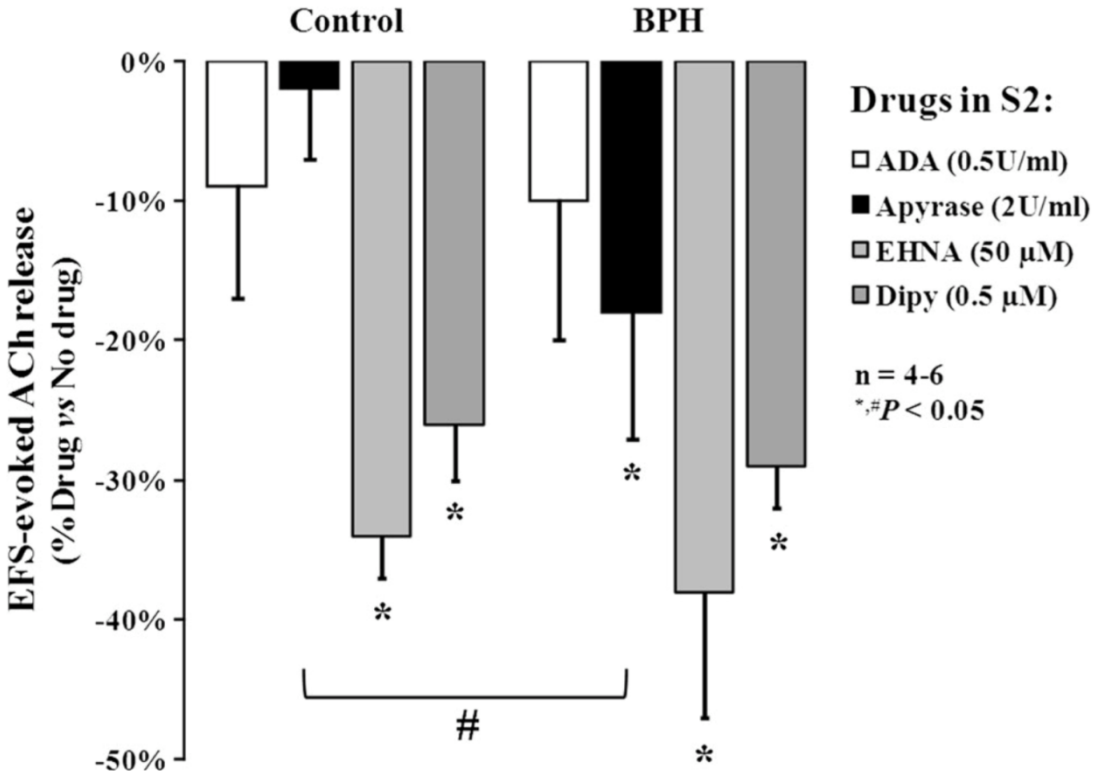

Figure 11 – Effects of adenosine deaminase (ADA), apyrase, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and dipyridamole (Dipy) on electrically-evoked [^{3}H]ACh release from urothelium-denuded detrusor strips from cadaveric organ donors (control) and BPH patients. ADA (0.5 U.mL^{-1}), apyrase (2 U.mL^{-1}), EHNA (50 µM) and Dipy (0.5 µM) were applied 15 min before S_2. The ordinates are changes in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained without addition of any drug. The data are means ± SD of four to six individuals. *,$^{\#}P$<0.05 (unpaired Student's *t*-test with Welch's correction) represents significant differences when compared with zero percent of change or with the effect of the same drug in control individuals, respectively.

Using mucosal-denuded human detrusor strips, we show here that in control conditions the ecto-NTPDase1/CD39 enzyme exerts a dominant role converting ATP directly into AMP, which is then sequentially hydrolyzed into adenosine and inosine by ecto-5'-nucleotidase/CD73 and ecto-adenosine deaminase, respectively. Despite the distribution of ectonucleotidase subtypes may present specificities among species and differ within the various layers of the bladder wall, immunofluorescence studies in the mouse showed that ecto-5'-nucleotidase/CD73 is present exclusively in the detrusor smooth muscle together with ecto-NTPDase1/CD39 (Yu et al., 2011). Data from a previous study

demonstrated that adenosine formation from extracellular ATP is negligible in isolated epithelial cells from the human urinary tract (Mohlin et al., 2009). This layout suggests that biosynthesis of adenosine from released ATP is positioned to favor a more important role of the nucleoside in suburothelial and detrusor muscle layers as compared to the urothelium where ecto-5'-nucleotidase/CD73 is almost absent if one excludes the basal cell layer (Yu et al., 2011); see also preliminary results in the human bladder in ref. (Correia-de-Sá, 2010).

Adenosine is a homeostatic metabolite in most organic systems mainly because it regulates neuronal excitability, vasodilation, smooth muscle relaxation, and release of vasoactive and neuroactive substances (Burnstock, 2014; Sheth et al., 2014); the nucleoside is also protective against ischemic and inflammatory insults. Therefore, we hypothesized that deficits in adenosine formation from released adenine nucleotides along with lifetime increments of ATP could contribute to detrusor overactivity in BPH patients. Deterioration of bladder neuromodulatory control has been hypothesized to explain the increase on nerve-evoked detrusor contractions in obstructed patients (Kosan et al., 2008). We show here that detrusor strips from BPH patients release higher amounts of ATP and ACh when stimulated electrically. The greater purinergic tone observed in detrusor strips from obstructed BPH patients (see also Bayliss et al., 1999) may be partially related to the impairment of ecto-NTPDase1/CD39 activity, thus limiting the breakdown of ATP released from bladder nerves and/or the urothelium; a situation that is in agreement with data from several pathological bladder conditions (Harvey et al., 2002). In this study, we expanded this concept by demonstrating that decreased activity of ectonucleotidases impacts on endogenous adenosine formation and, thus, on the inhibitory P1 receptors tonus of the human bladder. In parallel to the slower kinetics of the extracellular ATP catabolism, we demonstrated that AMP dephosphorylation leading to adenosine formation via ecto-5'-nucleotidase/CD73 was significantly decreased in bladder samples from obstructed BPH patients as compared to control individuals.

Several authors have shown that adenosine directly relaxes pre-contracted urinary bladder detrusor strips in different species (Acevedo et al., 1992; Brown et al., 1979; Burnstock et al., 1978a; King et al., 1997; Nicholls et al., 1992), including humans (Rubinstein et al., 1998). However, our results clearly indicate that adenosine-induced relaxation of detrusor contractions

requires high (unphysiological) millimolar concentrations of the nucleoside. On the contrary, our findings show that inhibition of nerve-evoked ACh release by adenosine and its stable analogues, NECA and R-PIA, was 30-times more potent than the relaxing effects of the nucleoside on ACh-induced detrusor contractions. Involvement of the A_1 receptor in the inhibitory action of adenosine and its analogues on transmitter release from the stimulated human detrusor was suggested by the selective blockade of their effects with DPCPX. Immunolocalization confocal microscopy data demonstrate for the first time that VAChT-positive cholinergic nerves are endowed with A_1 receptors, whereas the A_{2A} receptor is diffusely expressed on smooth muscle fibers of the human detrusor. A_1 receptor immunostaining is more intense in the detrusor of obstructed BPH patients as compared to control individuals, which may be caused in reaction to long-term deficits of adenosine formation from the catabolism of released ATP (see above). Thus, up-regulation of A_1 receptors on cholinergic nerves innervating the detrusor may contribute to explain the increased inhibitory sensitivity of nerve-evoked [^{3}H]ACh release to exogenous adenosine receptor agonists in bladders from BPH patients.

The way adenosine builds its influence to control cells communication depends on the extracellular concentration of the nucleoside, which is achieved by balancing extracellular formation and inactivation mechanisms, both cellular uptake via equilibrative nucleoside transporters and/or extracellular deamination into inosine by adenosine deaminase (Correia-de-Sá & Ribeiro, 1996; Gonçalves & Queiroz, 1993). Given the disparity between deficits in the adenosine-mediated tone (detected by the lack of effect of ADA on evoked [^{3}H]ACh release) and up-regulation of inhibitory A_1 receptors expression in the detrusor of BPH patients, we evaluated whether the A_1-receptor-mediated control of [^{3}H]ACh release could be rehabilitated by favoring adenosine accumulation with inhibitors of the nucleoside uptake system and adenosine deaminase. We concluded that both dipyridamole and EHNA might be useful for decreasing cholinergic nerve hyperactivity in patients with obstructed bladder due to BPH. Taking into consideration that there are differences in the potency of activation of pre-synaptic A_1 receptors on cholinergic nerves and A_{2A} receptors on smooth muscle fibers, prolongation of endogenous adenosine lifetime may be clinically safe

because it would hardly affect detrusor contractile tension that is essential to overcome outlet pressure during voiding in BPH patients.

Interestingly, *in vivo* cystometry experiments performed in the rat showed that both NECA and R-PIA administered intrathecally delayed the voiding reflex (Sosnowski & Yaksh, 1990). Moreover, prolongation of the intercontraction interval without affecting the amplitude of micturition was also observed when A_1 receptor agonists were applied into the lumen of the urinary bladder (Kitta et al., 2014). Overall, these findings suggest that adenosine controls the micturition cycle by acting predominantly on the nervous circuitry, both central and peripheral, without significantly affecting the voiding pressure that would result in urinary retention.

We are aware of possible limitations of the current study which may be attributed to age-related variance in the bladder cholinergic and purinergic tone among BPH patients (62±6 years of age) and the slightly younger control group (56±4 years of age). Although we did not explore the urological status of control men before harvesting the tissue, care was taken to prevent inclusion of individuals in the control group with past history of LUTS, as far as we could perceive from the clinical records of the intensive care unit and from interviewing close relatives. Yoshida et al. (2001) demonstrated age-related increases and decreases respectively in purinergic and cholinergic contractions of stimulated strips of the bladder of patients undergoing total cystectomy due to bladder carcinoma, but they also did not attempt to define if the patients had any kind of bladder dysfunction (Yoshida et al., 2001). We show here that electrical-stimulation of detrusor strips from BPH patients release 2.5-times more ATP than control preparations, which is in agreement with the purinergic neurotransmission changes detected in obstructed bladder patients using myographic recordings (Saito et al., 1997). Controversy however exists concerning the negative correlation between age and the cholinergic neurotransmission (Yoshida et al., 2004), because we observed a 1.5-fold increase rather than a decrease in evoked ACh release from the bladder of BPH patients, meaning that age-related changes cannot be the sole factor contributing to modifications in the bladder function among BPH patients and younger controls.

In conclusion, data from this study show for the first time that impairment of ecto-NTPDase1/CD39 activity unbalances extracellular ATP accumulation and endogenous adenosine formation leading to increased neuronal excitation in mucosal-denuded detrusor strips from BPH patients. While extracellular ATP accumulation may contribute to hyperexcitation of suburothelial nerve afferents via P2X3 receptors and to detrusor reactivity via P2X1 subunit-containing receptors, our study demonstrates that deficits in adenosine formation may also play a role in generating symptoms of bladder dysfunction in BPH patients. The loss of the inhibitory tone exerted by prejunctional A_1 receptors on ACh release from stimulated cholinergic nerves in the detrusor may, thus, be a target for therapeutic intervention of bladder dysfunctions associated with outflow obstruction due to BPH. Evidence for the therapeutic potential of adenosine A_1 receptor activation to decrease acetic acid-induced bladder overactivity has been recently gathered using anesthetized rats (Kitta et al., 2014).

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STATEMENT OF CONFLICTS OF INTEREST

None

ARTICLE 2

ATP FACILITATES NERVE-EVOKED ACETYLCHOLINE RELEASE FROM DETRUSOR STRIPS OF PATIENTS WITH BPH VIA P2X2/3 RECEPTORS ACTIVATION

Miguel Silva-Ramos[1,2,3,#], Isabel Silva[1,2,#], Miguel Faria[1,2] , Fátima Ferreirinha[1,2] and Paulo Correia-de-Sá[1,2]

[1]Laboratório de Farmacologia e Neurobiologia, [2]Center for Drug Discovery and Innovative Medicines (MedInUP), Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto, and [3]Serviço de Urologia, Centro Hospitalar do Porto (CHP), Porto, Portugal.

[#]MSR and IS contributed equally to this work

Author contribution:
Miguel Silva-Ramos: patients recruitment and selection, myographic recordings, acquisition, analysis and interpretation of experimental data, statistical analysis, drafting of the manuscript.
Isabel Silva: tissue processing/preservation and "in vitro" experiments, analysis and interpretation of experimental data, statistical analysis
Miguel Faria: myographic recordings, analysis and interpretation of data, statistical analysis.
Fátima Ferreirinha: immunofluorescence staining and confocal microscopy observation,, analysis and interpretation of imaging data.
Paulo Correia-de-Sá: conception and design of the study, project supervision, analysis and interpretation of experimental data, statistical analysis, revision of the manuscript.

ABSTRACT

Objective: This study was designed to investigate the role of ATP on cholinergic neurotransmission in human detrusor strips isolated from control individuals and from obstructed patients due to BPH.

Methods: Human detrusor samples were collected from 41 patients submitted to transvesical prostatectomy due to BPH and 26 male cadaveric organ donors. Urothelium-denunded detrusor strips were loaded with [^{3}H]-choline and the release of [^{3}H]ACh release was evoked by two periods of electrical field stimulation (10 Hz, 200 pulses of 1 ms duration). We also performed myographic recordings using isolated human detrusor strips to compare the magnitude of ACh- and ATP-induced bladder contractions in the two groups of individuals.

Results: Desensitization of ionotropic P2X1-3 receptors with the enzymatically-stable ATP analogue, α,β-MeATP (30 μM, applied for 15 min), significantly reduced the release of [^{3}H]ACh from stimulated detrusor strips by 14±6% and 52±7% in control individuals and BPH patients, respectively. Likewise, blockade of P2X receptors with PPADS (10 μM, a non-selective antagonist), TNP-ATP (10 nM, a preferential P2X2/3 antagonist) and A317491 (100 nM, a selective P2X3 antagonist) decreased nerve-evoked [^{3}H]ACh release by 6±8%, 27±9% and 20±2%, respectively, in control individuals. The inhibitory effects of PPADS (10 μM) and TNP-ATP (10 nM) were amplified to 43±6% and 43±6%, respectively, in the bladder of BPH patients, whereas A317491 (100 nM)-induced inhibition of transmitter release was kept unaltered (24±4%). Myographic recordings using epithelum-denuded detrusor strips showed that while the contractile activity of ACh (100 μM) decreased ($p<0.05$) from 4.74±0.46 mN (n=12) in control individuals to 2.64±0.27 mN (n=24) in BPH patients, ATP (3 mM)-induced contractions of the detrusor increased in BPH patients (1.13±0.24 mN, n=6) compared to controls (0.16±0.05 mN, n=6).

Conclusion: Data suggest that besides the well–characterized P2X1 receptor-mediated contractile activity of ATP in the human detrusor, endogenously released ATP facilitates the release of ACh from stimulated cholinergic nerve terminals through the activation of prejunctional P2X2/3 receptors. We also show here for the first time that the dual excitatory role of ATP at the cholinergic neuromuscular junction of the human bladder is exagerated in

obstructed patients due to BPH, leading us to hypothesize that it might contribute to detrusor overactivity and storage symptoms often seen in patients.

INTRODUCTION

Detrusor dysfunction associated with BOO can be a significant cause of LUTS. BOO can lead to detrusor overactivity and reduced bladder compliance causing storage-phase symptoms, and/or detrusor underactivity that causes further deterioration of voiding symptoms (Mirone et al., 2007). Detrusor overactivity has an huge impact on quality of life and can be found in half to two thirds of patients with clinically-dignosed BPH (Mirone et al., 2007). The fact that the prevalence of detrusor overactivity is reduced by 50% after prostatic surgery (Abrams et al., 1979), suggests that it is caused (at least in part) by obstruction. Despite this notion, the mechanisms underlying detrusor overactivity caused by obstruction are largely unknown (Andersson, 2003). Detrusor underactivity, which usually appears at the end stage of bladder dysfunction due to obstruction, is defined by the International Continence Society as a contraction of reduced strength and/or duration resulting in prolonged and incomplete emptying of the bladder. Although, a bit forgotten from the scientific literature, this aspect of the bladder physiology is gaining increased attention (van Koeveringe et al., 2011). Detrusor underactivity has been associated with age (Van Mastrigt & Rollema, 1992) and its prevalence is thought to be increasing due to ageing of the population. Until now, treatment is based on catheterization and there is no effective drug therapeutic available.

Nowadays, there is a considerable bulk of evidence supporting a role of purines in the pathophysiology of detrusor overactivity and in detrusor changes due to BOO (Burnstock, 2011). In humans, normal detrusor contraction is exclusively under cholinergic control since it is fully blocked by atropine (Fry et al., 1999). However, in hypertrophic bladders secondary to prostatic obstruction there is some degree of atropine resistance (Bayliss et al., 1999; Sjogren et al., 1982). Atropine-resistant contractions have been attributed to ATP, since they are blocked by desensitisation of ionotropic P2X receptors with the ATP stable analogue, α,β-methylene ATP (Bayliss et al., 1999). Despite these evidences, the importance of the purinergic component of detrusor contraction varies significantly between species (Ford et al., 2006; Wust et al., 2002) and changes in cholinergic and purinergic component in response to obstruction may differ substantially between rodents and man. In rodents and rabbits, the ATP-

mediated component has an important role in normal bladder contraction, but it does not seem to be exaggerated in obstructed (Banks et al., 2006) and neurogenic (Yokota & Yamaguchi, 1996) bladders, rendering these models less suitable for studying (purinergic) detrusor changes after BOO.

It has been attributed to ATP a prominent role in detrusor contractions in men with BPH, essentially by evoking more powerful smooth muscle contractions and by being credited its responsability for atropine-resistant contractions in these patients (Bayliss et al., 1999). This has been partially attributed to decreased ecto-ATPase activity (Harvey et al., 2002) leading to reduced inactivation and, thus, increased lifetime of ATP released by the urothelium and bladder efferent nerves. Purinergic component strengthening can also be explained by showing that ATP is released in higher amounts in obstructed than in control bladders, as we did in a recent report using urothelium-denuded detrusor strips stimulated electrically (Silva-Ramos et al., 2010; Silva-Ramos et al., 2015a).

Besides its action as a neurotransmitter/co-transmitter in several myoneural junctions, ATP can act presynaptically as a neuromodulator controlling the release of ACh and other neurotransmitters in several tissues (Duarte-Araújo et al., 2009; Ribeiro et al., 1996). The neuromodulatory role of ATP has been demonstrated in the bladder of rats and pigs (D'Agostino et al., 2012; King et al., 1997). In porcine detrusor stimulated electrically, ATP facilitated cholinergic neurotransmission via the activation of presynaptic P2X3 receptors (D'Agostino et al., 2012). These authors showed that the ATP-mediated neuromodulation was significantly increased by blocking the nucleotide breakdown with the ecto-ATPase inhibitor, ARL67156. In a recent report, our group showed that pathological deficits of detrusor ATP inactivation observed in BPH patients are responsible for extracellular ATP accumulation and decreased adenosine formation resulting in cholinergic hyperexcitation of the human bladder. While in the previous study we focused our attention on the deficient adenosine tonus and, subsequent, downregulation of A_1-mediated inhibition of ACh release (Silva-Ramos et al., 2015a), the pre-synaptic repercussion of surplus ATP accumulation at the cholinergic neuromuscular synapse remains to be determined in the human detrusor. In this context, the present study was designed to investigate the role of ATP on nerve-evoked ACh release from

human detrusor strips isolated from control individuals and from BPH patients. Filling this gap in our knowledge may provide novel targets for therapeutic intervention in detrusor overactivity and/or underactivity secondary to BOO.

MATERIALS AND METHODS

Tissue collection.

Samples of human detrusor were collected from the bladder dome of 41 patients with bladder outlet obstruction (BOO) due to BPH during transvesical prostatectomy (aged 71±7 years) and from 26 male cadaveric organ donors without known lower urinary tract pathology (aged 50±17 years). BOO and prostate enlargement were confirmed by uroflowmetry and ultrasonography, respectively. Collected samples were immediately placed at 4-6°C in mannitol transplantation solution at 400 mOsm/kg (M-400) not supplemented with ATP or adenosine (230 mM mannitol, 15 mM KH_2PO_4, 43 mM $K_2HPO_4 \cdot 3H_2O$,15 mM KCl, and 10 mM $NaHCO_3$, pH 7.4) and transported to the laboratory. Experiments were performed within the first 24 h after collection, which corresponds to the tissue viability window. This study and all its procedures were approved by the Ethics Committees of CHP and ICBAS-UP and were authorized by the National Transplantation Committee. All BPH patients signed an informed consent approved by the Ethics Committee of CHP for using the biological material. Regarding deceased organ donation, the legal frame work allows the "Presumed Consent" stating that residents in Portugal are consenting donors unless the individual previously objected during his or her life. The investigation conforms to the principles outlined in *The Code of Ethics of the World Medical Association* (Declaration of Helsinki).

[^{3}H]ACh release experiments.

Human detrusor strips (~1.5mm x 5mm) were mounted in vertical superfusion organ baths. The procedures used for labeling the preparations and measuring evoked [^{3}H]ACh release followed the previously described protocol with minor modifications (Correia-de-Sá et al., 1991). After a 30 min equilibration period, cholinergic nerve terminals were labeled for 40 min with 1 mM [^{3}H]-choline (specific activity 2.5 mCi nmol^{-1}) under electrical stimulation (EFS, 1-Hz frequency, 1-ms pulse width). Washout of the preparations was performed during

60 min, by superfusion (15 ml min^{-1}) with Tyrode's solution supplemented with choline uptake inhibitor, hemicholinium-3 (10 µmol L^{-1}). Tritium outflow was evaluated by liquid scintillation spectrometry (TriCarb2900TR, Perkin Elmer, Boston, USA) (% counting efficiency: 55±2%) after appropriate background subtraction, using 400-µL bath samples collected automatically every 3 min with a fraction collector (Gilson, FC203B, France). [^{3}H]ACh release was evoked by two periods of electrical field stimulation (S_1 and S_2, 200 pulses of 0.5 ms duration delivered at 10 Hz frequency). Therefore, the evoked [^{3}H]ACh release was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period (see *e.g.*Carneiro et al., 2014; Correia-de-Sá et al., 2006; Duarte-Araújo et al., 2004).

Test drugs were added 15 min before S_2 and were present until the end of the experiments. The change in the ratio between the evoked [^{3}H]ACh release during the two stimulation periods (S_2/S_1) relative to that observed in control situations (in the absence of test drugs) was taken as a measure of the effect of the tested drugs.

Myographic recordings.

Detrusor muscle strips without the mucosa were mounted in 10-mL capacity perfusion chambers connected to isometric force transducers. The changes in tension were recorded continuously with a PowerLab data acquisition system (Chart 5, v.4.2; AD Instruments, USA). Tissues were preloaded with 5 mN of tension and allowed to equilibrate for 90 min in Tyrode's solution, at 37ºC. Contractile responses were elicited by cumulative applications of ACh (0.1-100 µM) or ATP (0.01-3 mM).

Immunofluorescence staining and confocal microscopy observation

Detrusor samples fragments were fixed in PLP solution (paraformaldehyde 2%, lysine 0.075 M, sodium phosphate 0.037 M, sodium periodate 0.01 M) for 16 h at 4 ºC. Fixed tissue was cryoprotected with a solution containing 20% anhydrous glycerol dissolved in 0.1 M phosphate buffer, frozen, sectioned (16 µm) and incubated with a blocking buffer solution consisting in foetal bovine serum 10%, bovine serum albumin 1%, Triton X-100 0.3% in PBS, for 2 h with constant stirring. After blocking and permeabilization, samples were

incubated with rabbit anti-P2X1 primary antibody (1:50; Alomone #APR-001, Jerusalem, Israel) diluted in the incubation buffer (foetal bovine serum 5%, serum albumin 1%, Triton X-100 0.3% in PBS), at 4 ºC, for 16 h. After washing away unbound primary antibody, the sections were incubated with donkey anti-rabbit IgG secondary antibody (1:1000; Alexa fluor 488, #A-21206, Invitrogen) in the dark for two hours, at room temperature. Finally, tissue samples were mounted on optical-quality glass slides using VectaShield with DAPI as mounting media (VectorLabs) and stored in the dark at 4 ºC. Observations were performed and analyzed with a laser-scanning confocal microscope (Olympus FluoView, FV1000, Tokyo, Japan).

Drugs and Solutions.

Hemicholinium-3, choline chloride and pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS) were from Sigma (St Louis, MO, USA.); ATP, α,β-methylene ATP, 2',3'-*O*-(2,4,6-Trinitrophenyl)adenosine-5'-triphosphate tetra(triethylammonium) salt (TNP-ATP), 6-N,N-diethyl-d-β,γ-dibromomethylene ATP trisodium salt (ARL67156), 5-[[[(3-phenoxyphenyl) methyl] [(1S)-1,2,3,4-tetrahydro-1-naphthalenyl] amino] carbonyl]-1,2,4-benzenetricarboxylic acid sodium salt hydrate) (A317491), 8,8'-[carbonyl*bis*(imino-3,1-phenylenecarbonylimino)]*bis*-1,3,5-naphthalene-trisulphonic acid hexasodium salt (NF023) were obtained from Tocris Bioscience (Bristol, UK); [methyl^{3}H]choline chloride (in ethanol, 85.5 Ci.mmol-1) was from Perkin Elmer (Boston,USA); PPADS was made up as 3 mmol·L^{-1}, while TNP-ATP was made up as 10 mmol·L^{-1} stock solution in distilled water. A317491 was prepared in DMSO. All the other compounds were dissolved in Tyrode's solution. PPADS was kept protected from light to prevent photodecomposition. All stock solutions were stored as frozen aliquots at -20 ºC. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% v/v), were observed.

Presentation of data and statistical analysis

Results are expressed as mean ± SD, with *n* indicating the number of individuals used for a particular set of experiments. Only one experimental procedure (*e.g.* agonist in the absence and in the presence of the antagonist) was performed per individual. Statistical analysis of data was carried out using Graph Pad Prism 6.04 for Windows software (La Jolla, USA). Paired and unpaired Student's *t*-test with Welch's correction was used for statistical analysis when parametric data was considered. One-way analysis of variance (ANOVA) followed by the Holm-Sidak correction was used for multiple comparisons. Pearson r was used to test correlations. $P<0.05$ (two-tailed) values were considered statistically significant.

RESULTS

Effect of ATP on nerve-evoked [^{3}H]ACh release from human detrusor strips.

The release of [^{3}H]ACh induced by electrical field stimulation of human detrusor strips was significantly higher in samples from BPH patients (27.6±2.7 x 10^3 DPM.g^{-1} of wet tissue weight, *n*=12) than from control subjects (11.1±0.2 x 10^3 DPM.g^{-1} of wet tissue weight, *n*=14) (Figure 12). Desensitization of ionotropic P2X1-3 receptors with the enzymatically-stable ATP analogue, α,β-MeATP (30 μM, applied for 15 min), significantly ($p<0.05$) reduced the release of [^{3}H]ACh from stimulated detrusor strips by 14±6% (n=6) in control individuals and by 52±7% (n=4) in BPH patients (Figure 12). A similar inhibitory response pattern was obtained using the non-selective P2 receptor antagonist, PPADS (10 μM); this compound reduced [^{3}H]ACh release from stimulated detrusor strips by 6±8% (n=4) and 43±6% (n=4) in control individuals and BPH patients, respectively (Figure 13). While the selective P2X1 antagonist, NF023 (3 μM), was without effect, nerve-evoked [^{3}H]ACh release from human detrusor strips was also decreased by TNP-ATP (10 nM, a preferential P2X2/3 antagonist) and A317491(100 nM, a selective P2X3 antagonist) (Figure 13). The inhibitory effect of the selective P2X3 antagonist, A317491(100 nM), was roughly similar in detrusor strips from control individuals (20±2%, n=4) and BPH patients (24±4%, n=5), whereas TNP-ATP (10 nM) reduced more potently [^{3}H]ACh release from obstructed bladders (43±6%, n=4) than from control preparations (27±9%, n=4).

Alltogether these findings suggest that facilitatory P2X2/3 heteromers are upregulated on cholinergic nerve terminals of obstructed bladder strips, where they may be responsible for the increase in the purinergic tone underlying nerve-mediated detrusor overactivity.

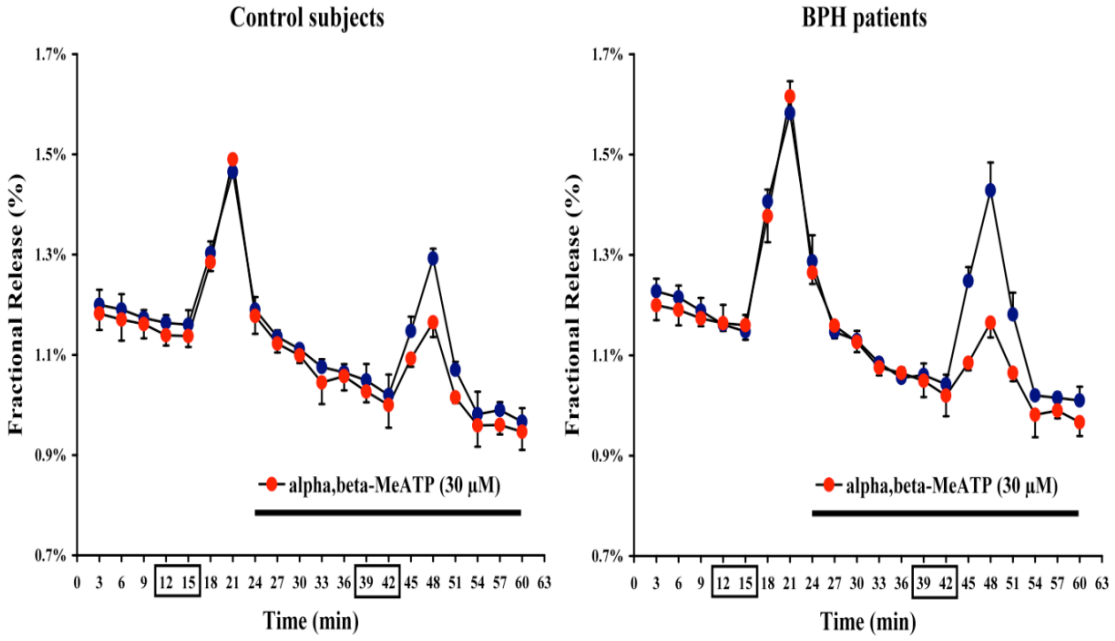

Figure 12. Desensitization of ionotropic P2X1-3 receptors with α,β-MeATP reduced the release of ^{3}H]ACh from stimulated detrusor strips from (A) control individuals and (B) BPH patients Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period (Fractional release, %) (see *e.g.*(Duarte-Araújo et al., 2004). Abscissa indicates the times at which samples were collected. [^{3}H]ACh release was elicited by electrical field stimulation (10 Hz, 200 pulses of 0.5 ms duration) twice, starting at 12th (S_1) and 39th (S_2) minutes after the end of washout (zero time). αβ-MeATP(30µM, red dots) was added to the incubation media 15 min before S_2 (horizontal bar); for comparison purposes, we show a time course of tritium outflow in control conditions (blue dots) where no drug was added during the collecting period. The vertical bars represent SD of four (A) to six (B) different individuals. Note that the spontaneous tritium outflow was not changed in the presence of αβ-MeATP(30µM)

Contractile responses of isolated human detrusor strips to ACh andATP.

Bath application of acetylcholine (ACh, 0.01-100 µM) concentration-dependently increased smooth muscle tension in the detrusor of control organ

donors, but cholinergic-induced contractions had a smaller amplitude in samples from patients with bladder outlet obstruction due to BPH (Figure 14A). For instance, the contractile activity of ACh (100 μM) decreased ($p<0.05$) from 4.74±0.46 mN (n=12) to 2.64±0.27 mN (n=24) in control individuals and BPH patients, respectively. To exclude bias due to age difference betweeen BPH patients (71±7 years) and control organ donors (50±17 years), we performed a correlation analysis between age and the magnitude of ACh (10 μM)-induced contractions; we obtained a Pearson's *r* value of -0.048, which did not reach statistical significance (p=0.810).Therefore, ageing does not seem to contribute, on its own, to decrease the sensitivity of detrusor smooth muscle cells to ACh.

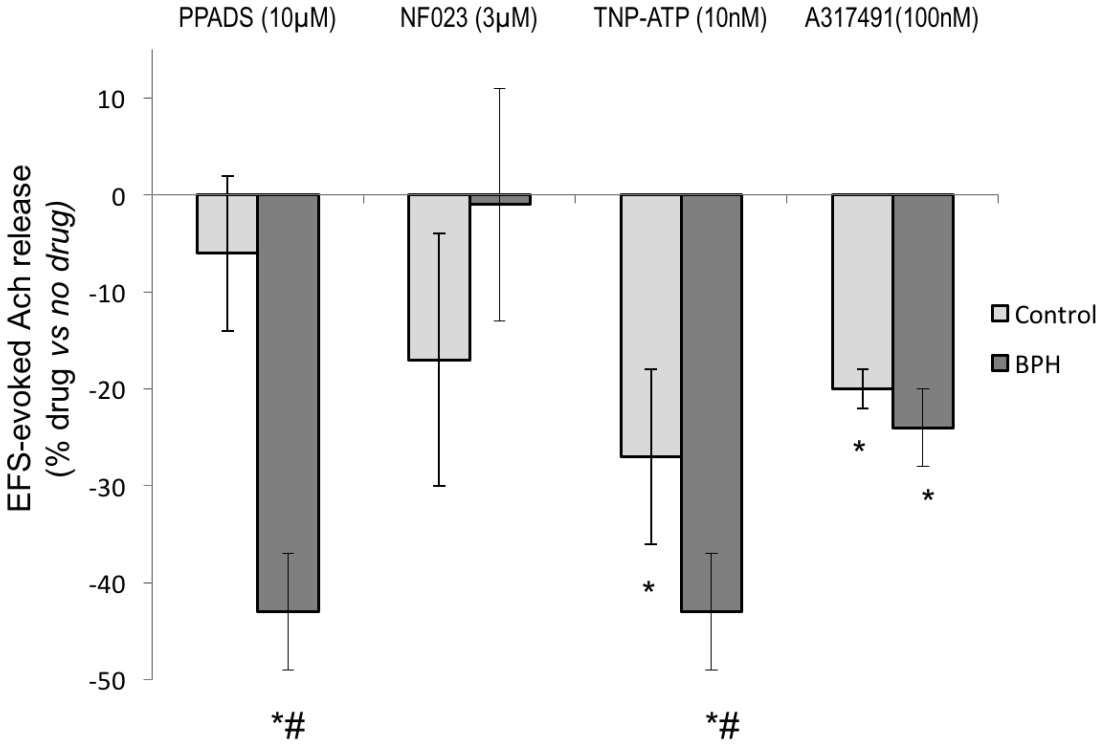

Figure 13. Effects of P2X receptor antagonists on electrically-evoked [^{3}H]ACh release from human detrusor strips from cadaveric organ donors (control) and BPH patients..PPADS (10 μM), NF023 (3 μM), TNP-ATP (10 nM) and A317491 (100 nM) were applied 15 min before S_2. The ordinates are changes in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained without addition of any drug. The data are means ± SD of four to six individuals. *,#*P*<0.05 (unpaired Student's *t*-test with Welch's correction) represents significant differences when compared with zero percent of change or with the effect of the same drug in control individuals, respectively.

In aggreement with data in the literature, ATP (0.01-3 mM) was virtually devoid of effect on detrusor strips from control individuals (Figure 14A), but the nucleotide concentration-dependently increased (*P*<0.05) the tension of detrusor

strips isolated from BPH patients. At the maximal concentration tested, ATP (3 mM)-induced detrusor contractions in BPH patients reached 40% (1.13±0.24 mN, n=6) of the effect caused by 100 μM ACh and this value was significantly ($p<0.05$) higher than that observed in the strips from control individuals (0.16±0.05 mN, n=6) (Figure 14A).

The competitive inhibitor of human NTPDase1 (K_i 11±3 μM) and NTPDase3 (K_i 18±4 μM), ARL67156 (100 μM), significantly ($p<0.05$) potentiated the contractile effect of ATP (0.1-3 mM) on detrusor strips isolated from BPH patients (Figure 14B).

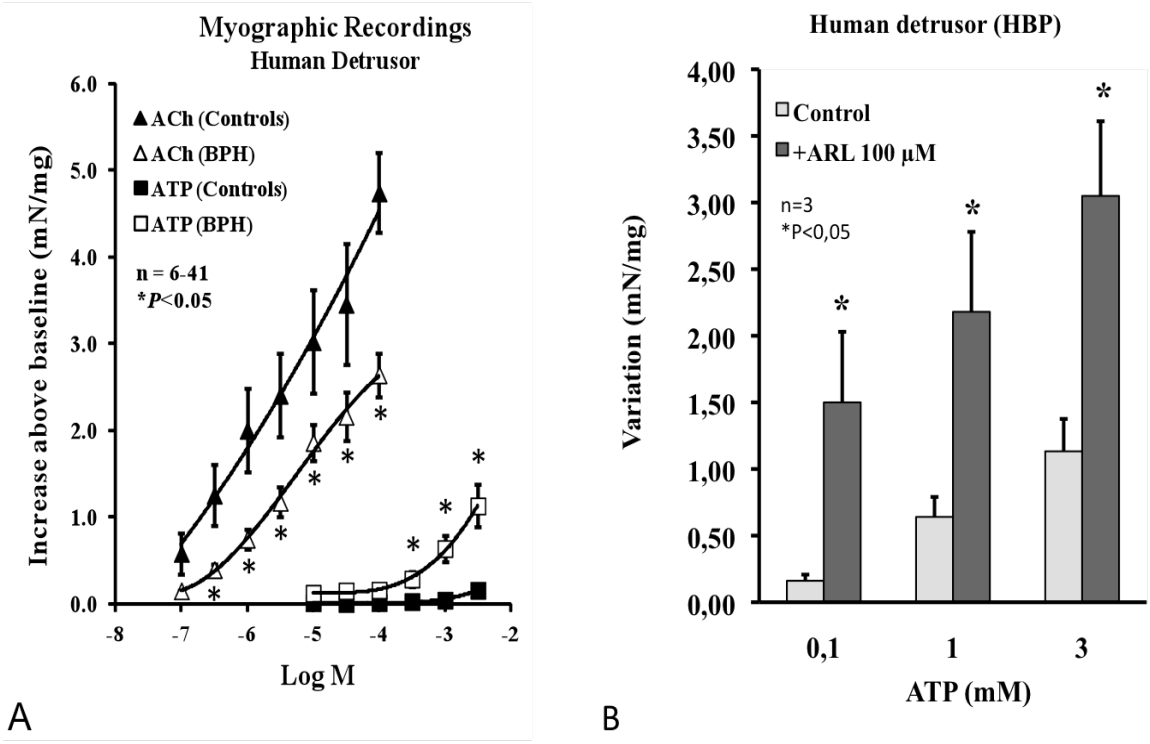

Figure 14. A. Contractile responses of isolated human detrusor strips from control individuals and BPH patients to ACh (0.1-100 μM) andATP (0.01-3 mM). The vertical bars represent SD of an *n* number of individuals. **P*<0.05 (unpaired Student's *t*-test with Welch's correction) represent significant differences when compared to control organ donors. **B. Pretreatment with the NTPDase inhibitor, ARL67156 (100 μM), potentiated ATP (0.1-3mM)-induced contractions of detrusor strips isolated from BPH patients.** Bars represent mean±SD of three individuals. **P*<0.05 (unpaired Student's *t*-test with Welch's correction) represent significant differences when compared to the the effect of ATP alone.

These results strenghten our previous assumption that NTPDase1/CD39 may be the main responsible for ATP/ADP inactivation in the human detrusor (Silva-Ramos et al., 2015a), also taking into consideration that ARL67156 has only marginal effects on NTPDase2, NTPDase8 and ecto-5′-nucleotidase/CD73

activities (Levesque et al., 2007). Involvement of ATP-preferring NTPDase3 is also possible, but this enzyme exhibits an intermediate pattern of product formation leading to a transient accumulation of diphosphonucleosides (Robson et al., 2006), which we did not observe in the enzymatic kinetic experiments performed in the human detrusor using ATP as substrate (Silva-Ramos et al., 2015a; see also Chapter 3 / Article 1). Interestingly, the potentiating effect of ARL67156 (100 μM) on ATP-induced contractions was observed in detrusor samples from obstructed patients even though this pathological condition has been associated to partial impairment of ecto-NTPDase1/CD39 activity (Harvey et al., 2002).

The detrusor of BPH patients express higher levels of P2X1 receptors than control individuals

Immunofluorescence confocal microscopy studies demonstrate the presence of P2X1 receptors on the human detrusor. The immunolabeling pattern shows that P2X1 receptors are distributed along the plasma membrane of smooth muscle fibers (Figure 15)(see also (Elneil et al., 2001). Data also show that detrusor samples from BPH patients exhibit higher amounts of P2X1 immunoreactivity compared to control individuals (Figure 15), which is in agreement with our functional results and with RT-PCR studies (O'Reilly et al., 2001).

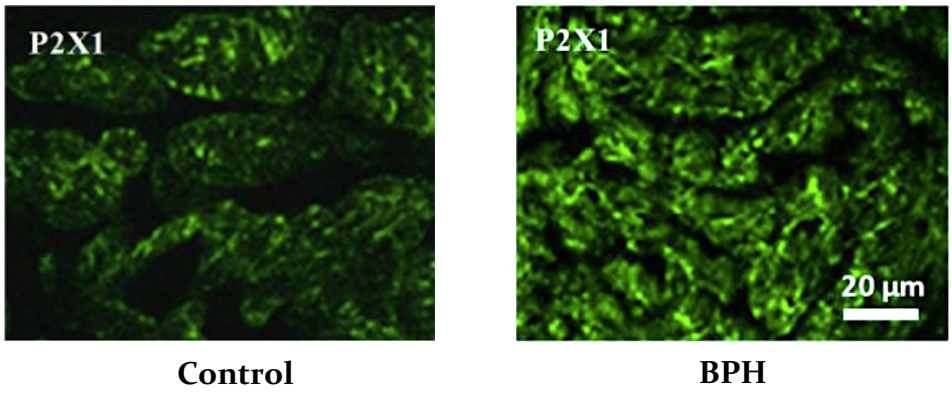

Figure 15. Confocal micrographs showing the P2X1 receptor immunoreactivity (green fluorescence) in the detrusor of a cadaveric organ donor (Control) and a patient with BOO (due to BPH). Similar results were obtained in 4 additional experiments. Scale bar = 20 μm.

DISCUSSION

In a previous study, we showed that cholinergic neurotransmission was increased in mucosal-denuded detrusor strips from BPH patients compared to control organ donors and that this could be due, at least in part, to deficits in the activity of ecto-NTPDase1/CD39 subsequently unbalancing extracellular ATP accumulation and endogenous adenosine formation (Silva-Ramos et al., 2015a; see also Chapter 3 / Article 1). Here, we show that extracellular ATP accumulation increases bladder tension in obstructed patients by two synergistic mechanisms. One, is mediated through direct activation of ionotropic P2X1 receptors which are more expressed on smooth muscle fibres of obstructed human bladders. The second one, is operated indirectly through facilitation of ACh release from stimulated cholinergic nerve efferents via the activation of P2X2/3 receptor heteromers sensitive to PPADS, TNP-ATP and A317491 antagonists and to desensitization by the enzymatically-stable ATP analogue, α,β-MeATP. The unbalance between the excessive ATP-mediated P2X2/3 receptor facilitation of ACh release and the loss of the adenosine A_1-receptor-mediated inhibitory tone of cholinergic nerve terminals contributes to increase detrusor reactivity in obstructed patients with BPH. While the purinergic-related detrusor overactivity may be a burden for patients with BOO, which is certaninly worth to be explored therapeutically using P2X receptor antagonists and/or adenosine A_1 receptor modulators (e.g. receptor agonists, uptake and deaminase inhibitors), its maintenance/potentiation in later stages of the disease may be attractive to overcome detrusor underactivity. Even though we did not explore specifically this possibility, we could increase ATP-mediated contractions of detrusor strips from BPH patients using a competitive inhibitor of human NTPDase1/CD39, ARL67156 (100 μM). Therefore, we hypothesize that manipulation of the purinergic tone is even more relevant to overcome detrusor underactivity taking into consideration that cholinergic activity is substantially impaired in patients with BOO due to BPH (see below).

The effect of BOO on the release of ACh was only previously studied in animals. In a rat model of obstruction, the release of ACh (per weight of wet tissue) from stimulated detrusor strips decreased in parallel with the reduction in the density of nerves innervating the bladder (Murakami et al., 2008). However, density changes must take into account that obstructed bladders exhibit

hypertrophy of the smooth muscle cells and substantial intercellular deposition of collagen and elastic fibres, which caused a 4-fold increase in bladder versus body weight ratio. Therefore, if one corrects the amount of ACh release by the increase in bladder weight secondary to obstruction in the Murakami study, we easily reach the conclusion that the absolute amount of ACh released in response to electrical stimulation is similar (or even higher) in obstructed than in control rat bladders and the same certainly occurs with the number of nerves existing in the bladder of the two groups of animals. Moreover, experimental evidences agree that animal models of bladder outlet obstruction do not exactly mimic the pathological features observed in BOO caused by BPH in men; obstruction in animal models generates very rapidly rigid bladders, whereas the bladder of obstructed BPH patients is relatively elastic and this pathological feature develops progressivley during several years. As a matter of fact, the bladder of BPH patients changes gradually resulting in increases in the bladder mass less than 100% of the original weight (Kojima et al., 1996), which is far from the 4-fold bladder weight gain observed by the Murakami group as soon as 2 weeks after surgery to partially oclude bladder outlet in the rat (Murakami et al., 2008); these findings were reproduced by other authors who observed a 6.3-fold increase in bladder weight due to detrusor hypertrophy 6-weeks after surgical obstruction of the rat bladder (Hampel et al., 2002). Rapid development of smooth muscle hypertrophy and intersticial fibrosis supports for the inverse relationship between detrusor contractility and degree of outlet obstruction in obstructed animal models (Austin et al., 2004; Moore et al., 2002) and may explain why the detrusor decompensates so early in the course of experimental obstruction (Michel & Barendrecht, 2008), which is a situation that is observed only at later stages of the human disease. Investigation of the mechanisms underlying species differences in reaction to BOO, namely the changes in detrusor morphology and functional activity, is beyond the scope of the present study but it certainly deserves future attention as they might prove useful to the discovery of novel therapeutic targets to preserve detrusor function during the course of the human disease.

Previous studies using obstructed human bladder samples admitted that this pathological condition was associated with a partial bladder denervation compared to control specimens (Gosling, 1997; Harrison et al., 1987).

Unfortunately, these authors did not directly assess cholinergic innervation of the human bladder and, most importantly, they also failed to actually measure bladder denervation, *i.e.* reduction in the number of nerve fibres. As criticised above regarding animal studies, a decrease in the density of nerve fibres within the bladder wall is not an index of bladder denervation because one must take into account smooth muscle hypertrophy and more or less extensive interstitial deposition of extracellular matrix proteins (e.g. collagen, elastin), which moves apart nerve fibres and tiny axon terminals. The partial denervation hypothesis was also sustained by functional studies showing that muscle biopsies from patients with detrusor instability due to BOO demonstrated supersensitivity to ACh and reduction in nerve-mediated responses compared with strips from stable bladders (Harrison et al., 1987). Yet, in this respect, functional studies are not consensual (Michel & Barendrecht, 2008; Turner & Brading, 1997). We are aware that discrepancies among detrusor responsiveness to ACh in BPH versus control patients may be a consequence of age differences between the two groups (Mansfield et al., 2005; Yoshida et al., 2001). We excluded this possibility because in our study we detected no significant correlation between age and the magnitude of ACh-induced detrusor contractions.

Notwithstanding this dispute, we found that electrical field stimulation of utothelium-denuded detrusor strips from obstructed BPH patients release significantly ($p<0.05$) higher amounts of [^{3}H]ACh than the samples isolated from control organ donors. Thus, if the partial denervation hypothesis is confirmed, our data suggest that the amount of ACh released per nerve unit must be highly enhanced in the bladder of BPH patients. Interestingly, we showed here that the amplitude of ACh-induced contractions was significantly attenuated in obstructed BPH bladders compared to control organ donors and similar situation was observed regarding carbachol-induced contractions in rats tested 6 months after surgery to cause BOO (Murakami et al., 2008). Reduction of cholinergic sensitivity of detrusor smooth muscle fibres may attenuate nerve-evoked contractions of obstructed human bladders, even though ACh release is being released in excess from activated cholinergic nerve efferents. All these nuances of the cholinergic neurotransmission must be taken into account before drawing any conclusion from the analysis of separate experimental data.

More importantly, our results suggest that anticholinergic agents may be less effective in treating detrusor overactivity in BPH patients. Since the majority of these compounds are competitive muscarinic antagonists blocking preferentially M_3 receptors on smooth muscle fibres, their action may be substantially hampered in obstructed BPH patients because ACh release is facilitated leading to increased levels of the transmitter in the synaptic cleft and/or muscarinic receptors activity is downregulated by excessive cholinergic exposure, as it has also been demonstrated in the BOO rat (Braverman & Ruggieri, 2003). It, thus, appears that net cholinergic neurotransmission in obtructed bladders from BPH patients is compensated by the increase in ACh release from activated nerve terminals, but this does not fully explain detrusor overactivity often seen in BPH patients unless one hypothesizes the release/accumulation of a second extracellular messenger, like ATP, responsible for increasing the activity of cholinergic nerve efferents and detrusor activity. According to this theory, blockade of purinergic signals may be a valuable alterative worth to test for treating detrusor overactivity in BPH patients resistent to antimuscarinics.

The role of ATP in the process of enhancing detrusor contraction in obstructed patients seems pivotal. ATP has long been considered as a “damage signaller” in several systems and the nucleotide has important roles in tissue remodelling secondary to injury (Burnstock & Verkhratsky, 2010). The exact mechanism by witch the purinergic signalling cascade is activated in the detrusor of BPH patients is not yet established. There is a considerable bulk of evidence showing that BOO causes tissue ischemia (Azadzoi et al., 1996; Belenky et al., 2003; Saito et al., 1997; Yamaguchi et al., 2014) and this may trigger the release of purines from affected cells. Experimentally-induced ischemia secondary to hypoxia-reoxygenation cycles increases atropine-resistant nerve-mediated contractions in the rat detrusor, an effect that can be blocked by P2X antagonists (Elliott et al., 2013). Furthermore, ischemia lowers tissue pH, which is usually associated with deficits in the activity of NTPDases leading to extracellular ATP accumulation (Kukulski et al., 2005) along with the upregulation of ionotropic P2X receptors expression and signalling (Zhang et al., 2014). Although we did not address this issue in this study, ischemia-induced purinergic signalling

upregulation may be a mechanism worth to explore in the future in order to explain the pathophysiology of detrusor overactivity in BPH patients.

We show here for the first time that endogenously released ATP facilitates ACh outflow from utothelium-denuded human detrusor strips and this effect seems to be significantly amplified in obstructed BPH patients. Facilitation of ACh release by ATP has been previously shown in other synapses (Magalhães-Cardoso et al., 2003; Sperlágh et al., 2007). Our results suggest that ATP-mediated facilitation of ACh release is dependent on the activation of ionotropic P2X1-3 receptors, as these receptor subtypes are the only amenable to desensitization by α,β-methylene ATP. Similar results were obtained in the porcine detrusor (D'Agostino et al., 2012); these authors showed that that short-term administration of ATP or α,β-methylene ATP significantly increased ACh release, but the facilitatory effect disappeared upon prolonging the time of incubation with the two compounds. In contrast to the well-known contractile effect of ATP, via P2X1 receptors activation, in the human detrusor, this receptor subtype does not participate in ATP neuromodulation. This was concluded because the selective P2X1 receptor antagonist, NF023, was devoid of effect on nerve-evoked ACh release from detrusor strips of control organ donors and BPH patients. Blockade of P2X2/3 heteromers with TNP-ATP, as well of the P2X3 receptor with its selective antagonist, A317491, significantly decreased the release of ACh from stimulated detrusor strips of control individuals. Interestingly, the inhibitory effect of TNP-ATP almost doubled its magnitude in samples collected from obstructed BPH patients, whereas the effect resulting from selectively blocking the P2X3 receptor with A317491 remained unaltered. These findings suggest that both P2X2/3 and P2X3 receptors may be involved in ATP-mediated facilitation of nerve-evoked ACh in the human bladder, but enhancement of the purinergic neuromodulation detected in bladders of obstructed BPH patients might result from the upregulation of the P2X2/3 receptor subtype. The presence of P2X3 receptors and P2X2/3 heteromers has been detected in peripheral nerves by immunofluorescence microscopy (Dunn et al., 2001). These receptors have been found in suburothelium nerve fibers of the human bladder (Brady et al., 2004) and their presence was also detected in nerve bundles of rat and human detrusors (Lee et al., 2000; Neuhaus et al., 2012).

In conclusion, data produced here and in previous studies support the idea that in the bladder of BPH patients several mechanisms are activated towards the generation of stronger detrusor contractions, including (1) deficits in the metabolism of ATP leading to (2) increased contractile responses mediated by post-junctional P2X1 receptors, (3) activation of facilitatory ionotropic P2X2/3 receptors and decreased adenosine A_1-receptor-mediated inhibitory tonus on cholinergic nerves that end-up to (4) facilitate ACh release from stimulated nerve terminals, which (5) partially compensates the lack of sensitivity of cholinoceptors present on smooth muscle fibres. Recent papers have pointed out, that P2X3 (and/or P2X2/3) antagonists could be potential candidates for the treatment of painful bladder and OAB syndromes (Ford & Undem, 2013; Smith, 2013). Besides the relevance of P2X3 receptors in mediating bladder sensations, our data add important information concerning the implication of these receptors on cholinergic neuroexcitability and detrusor overactivity that must be also accounted. Our results also shed light on possible ways to increase bladder performance in situations of detrusor underactivity resulting from BOO. Until now there is no pharmacological treatment for detrusor underactivity and this is a clear unmet medical need (Osman et al., 2014). It is appealing the prospect of enhancing detrusor performance in these patients through the activation of presynaptic P2X2/3 receptors, either by using subtype-specific receptor agonists or by inhibiting the activity of NTPDase1/CD39, as we did with ARL67156.

ACKNOWLEDGEMENTS

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STATEMENT OF CONFLICTS OF INTEREST

None

3.2 URINARY ATP IN HEALTH AND DISEASE

What's in a name: "purine" comes from "urine"!

ARTICLE 3

INCREASED URINARY ADENOSINE TRIPHOSPHATE IN PATIENTS WITH BLADDER OUTLET OBSTRUCTION DUE TO BENIGN PROSTATE HYPERPLASIA

Miguel Silva-Ramos[1,2], Isabel Silva[1], José Carlos Oliveira[3]and Paulo Correia-de-Sá[1]

[1]Laboratório de Farmacologia e Neurobiologia, Center for Drug Discovery and Innovative Medicines (MedInUP), Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto, [2]Serviço de Urologia and [3]Serviço de Química Clínica, Centro Hospitalar do Porto (CHP), Porto, Portugal.

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Author contribution:
Miguel Silva-Ramos: conception and design of the study, patients selection and recruitment, analysis and interpretation of data, statistical analysis, drafting of the manuscript.
Isabel Silva: samples processing/preservation, ATP and LDH determinations, analysis and interpretation of data, statistical analysis
José Carlos Oliveira: biochemistry and urine analysis.
Paulo Correia-de-Sá: conception and design of the study, project supervision, analysis and interpretation of experimental data, statistical analysis, revision of the manuscript.

ABSTRACT

Background: Diagnosis of bladder outflow obstruction in patients with lower urinary tract symptoms is challenging without using invasive urodynamic tests. Recently, we showed *in vitro* that urothelial strips from patients with BPH release more ATP than controls. Here, we tested whether urinary ATP can be used as a wall tension transducer non-invasive biomarker to detect BOO in patients with BPH.

Methods: 79 male patients with BOO and 22 asymptomatic controls were recruited

prospectively. Patients were asked to complete the International Prostate Symptom Score (IPSS) questionnaire and to void at normal desire into a urinary flowmeter, the postvoid residual volume was determined by suprapubic ultrasonography. Urine samples from all individuals were examined for ATP, creatinine and lactate dehydrogenase,

Results: BOO patients had significantly higher ($p<0.001$) urinary ATP normalized by the voided volume (456±36 nmol) than age-matched controls (209±35 nmol). Urinary ATP amounts increased with the voided volume, but the slope of this rise was higher in BOO patients than in controls. A negative correlation was detected between urinary ATP and flow rate parameters, namely maximal flow rate ($r=-0.310$, $p=0.005$), Siroky flow-volume normalization ($r=-0.324$, $p=0.004$), and volume-normalized flow rate index ($r=-0.320$, $p=0.012$). We found no correlation with LUT symptoms IPSS score. Areas under the receiver operator characteristics (ROC) curves were 0.91 (95% CI 0.86-0.96, $p<0.001$) for ATP alone and 0.88 (95% CI 0.81-0.94, $p<0,001$) when adjusted to urinary creatinine.

Conclusions: Patients with BOO release higher amounts of ATP into the urine than the control group. The high area under the ROC curve suggests that urinary ATP can be a high-sensitive non-invasive biomarker of BOO, which may have a discriminative

value of detrusor competence when comparing BPH patients with low urinary flow rates.

INTRODUCTION

Lower urinary tract symptoms are very frequent in adult men. The etiology is multifactorial. Although BOO due to benign prostatic hyperplasia is a common cause of LUTS, bladder dysfunction and disorders outside the urinary tract can cause these symptoms as well (Gratzke et al., 2015). European studies estimated a prevalence of about 50% of BOO in patients with LUTS due to BPH (Ockrim et al., 2001; Oelke et al., 2007).

One of the challenges clinicians face when evaluating patients with LUTS due to BPH is to determine the existence of BOO. Diagnosing obstruction is vital to establish a proper therapeutic plan and define prognosis. Although medical history and physical examination are paramount they are subjective and more objective complimentary tests are usually recommended (D'Silva et al., 2014). Uroflowmetry and ultrasound imaging focusing on the evaluation of bladder wall thickness, prostatic volume, intravesical prostatic protrusion and postvoid residual volume (PVR) allow gross estimation of the degree of obstruction. However these methods are not reliable due to inter-observer variability, low accuracy and lack of standardization (Oelke, 2010; Oelke et al., 2007). As a matter of fact, obstruction is considered an urodynamic concept that is characterized by increased detrusor pressure and decreased urinary flow rate during voiding, justifying why the pressure flow study (PFS) is considered the gold-standard method for this diagnosis. Its invasive nature, limited access and high cost are major obstacles for using this test in routine clinical practice. Several authors have been trying to design novel non-invasive methods for measuring detrusor pressure with limited success, which are still considered experimental (Elterman et al., 2013).

ATP is known to be released by the urothelium in response to mechanical stimuli (Ferguson et al., 1997; Vlaskovska et al., 2001). Once bladder filling reaches a threshold of vesical pressure, ATP released from urothelial cells may activate P2X3 receptors located in suburothelial nerve fibers that convey sensory information to the central nervous system (Birder, 2006; Cockayne et al., 2000). Ussing chamber experiments demonstrated that the amount of ATP released to the luminal side of the urothelium is 50-fold higher than that detected in the serosal side in response to similar stimulation conditions (Wang et al., 2005).

Moreover, we and others provided evidences showing that the ATP catabolism by ectonucleotidases is faster in the serosal compared to the luminal side of the urothelium (Silva-Ramos et al., 2015b; Wang et al., 2005). This favors ATP accumulation in the bladder lumen, which can be detected in urine samples and voided urodynamic fluid. For instance, the amount of ATP measured in the urodynamic fluid instilled during cystometry inversely correlated with first desire to void in women with OAB (Cheng et al., 2013; Cheng et al., 2010). Our own data show that urinary ATP measured during voiding at normal desire is significantly higher in women with detrusor overactivity compared to age-matched controls (Silva-Ramos et al., 2013b). These findings agree with cystometry experiments in freely moving rats showing that intravesical ATP stimulates the micturition reflex (Pandita & Andersson, 2002).

Besides this association between urinary ATP and bladder sensitivity, ATP can also be seen like a pressure transducer because urothelial cells respond to tension-induced deformation by releasing ATP (Ferguson et al., 1997). As per the Laplace's Law stating that wall tension is proportionate to the pressure times half of the radius of a spherical vessel, we hypothesized that urinary ATP amounts would be significantly higher in patients with BOO as a consequence of increased bladder pressure during voiding in a similar manner to that occurring in patients with detrusor overactivity (Kumar et al., 2010; Silva-Ramos et al., 2013b). In support of this theory, we recently showed that electrical stimulation of mucosal strips containing only the urothelium with the attached lamina propria from BPH patients increased the isometric tissue tension above the spontaneous phasic activity and released five times more ATP than the control preparations (Silva et al., 2015). These findings clearly indicate that besides urothelium stretching during bladder filling the isometric increase in transmucosal pressure can also contribute to ATP release in the human bladder. In order to translate *in vitro* experimental findings to the clinical practice, this study was designed to test if patients with BOO due to BPH have higher urinary ATP amounts than asymptomatic controls and whether measurement of urinary ATP is reliable to determine non-invasively the existence of increased bladder pressure due to BOO.

METHODS

Patients and procedures.

This study and all its procedures were approved by the Ethics Committees of Centro Hospitalar do Porto (CHP) and of Instituto de Ciências Biomédicas de Abel Salazar (Medical School) of the University of Porto (ICBAS-UP). All subjects signed an informed consent prior to examinations and for using the biological material. The investigation conforms to the principles outlined in the Declaration of Helsinki. A total of 101 men were enrolled in this study, including 79 patients with BOO (40-79 years of age, mean 66±9 years) and 22 asymptomatic volunteers (40-75 years of age, mean 53±11 years) recruited among hospital staff and patients' attendants from the orthopedics clinic. Exclusion criteria of volunteers included any LUTS, any urinary tract infection, any intervention on lower urinary tract, malignancy, neurological disease (e.g., diabetes mellitus, prolapsed of intravertebral discs, spinal trauma, etc.), or medications known to affect the lower urinary tract function (e.g., anticholinergics, alpha blockers, adrenergic stimulants, antibiotics, etc.). Patients were enrolled between March 2012 and February 2015 from the waiting list for prostatic surgery to relieve BOO due to BPH of the Department of Urology of CHP. The indication for surgery was the responsibility of the attending urologist. Patients with history of malignancy of any sort (including bladder and/or prostate cancer), pelvic radiotherapy, neurologic disease, any systemic or inflammatory condition, active urinary tract infections, renal impairment (Chronic Kidney Disease Stage > 2) or indwelling urinary catheterism, were excluded. Patients were asked to complete a Portuguese version of the International Prostate Symptom Score (IPSS) questionnaire and to void comfortably at normal desire in a standing position into a gravimetric urinary flowmeter (Flowmaster, MMS, Enschede, The Netherlands). Adequate privacy was provided to each participant to minimize psychological inhibition. Voided volume was recorded and postvoid residual volume was estimated by suprapubic ultrasonography. Voids under 100 ml and over 500 ml were excluded from the comparative analysis in order to curtail extreme variations among the two study groups and because accurate representation of maximal flow rates in Siroky flow-volume normograms requires a minimum voided volume (Siroky et al., 1979). Urine samples obtained from natural voids were used for rapid dipstick test and urine culture. Subjects testing positive for nitrites,

leucocytes or infection, were also excluded. A third urine sample was aliquoted, snap frozen in liquid nitrogen and preserved at −80°C until ATP determination. These samples were also used to measure urinary creatinine (Cr) and lactate dehydrogenase (LDH, EC 1.1.1.27) activity (see *e.g.* Silva-Ramos et al., 2013b). LDH is a fairly stable intracellular enzyme that is widely used as an indicator of cell integrity providing that its values are kept at a low level. ATP values were normalized to urinary volume and creatinine in order to reduce sample variation and to discard kidney influence on ATP amounts. All biochemical tests were done blindly. Data on prostatic volume and prostate-specific antigen (PSA) were collected from patient's clinical records.

Measurement of urinary ATP.

Undiluted urine samples were defrosted till 25°C and afterwards centrifuged at 3000 g at room temperature for 20 seconds to remove cellular debris. The supernatant was separated. A mixture of luciferin-luciferase was added according to the manufacturer instructions using the ATP Bioluminescence Assay Kit HS II (Roche Applied Science Indianapolis, Indiana, USA). ATP detection was evaluated using a multi-mode microplate reader (Synergy HT, BioTek Instruments Inc., Vermont, USA) controlled via BioTek's Gen5™ Data Analysis Software. Sample bioluminescence was compared to that of standard amounts of ATP used in the same concentration range; standard ATP samples were prepared daily (*cf.* Silva-Ramos et al., 2013b). All samples were run in duplicate. The minimum ATP detection limit in these experimental conditions was 10^{-12} M (10^{-16} moles in 100 µl samples) and luminescence correlated linearly to ATP concentration till 10^{-6} M. Dilution of urine samples were done whenever necessary in order to fit interrogation values in the linear part of the ATP calibration curve.

Measurement of urinary creatinine and LDH.

Each urine sample remaining from ATP measurements was used to quantify urinary creatinine and the lactate dehydrogenase (LDH, EC 1.1.1.27) activity (Silva-Ramos et al., 2013b). The quantitative determination of urinary creatinine was performed *in vitro* on a Cobas Integra 800 analyser using the kinetic colorimetric Creatinine Jaffé Gen.2 assay according to the manufacturers'

instructions (Roche Diagnostics GmbH, Mannheim, Germany). LDH is an oxidoreductase enzyme that catalyses the interconversion of pyruvate and lactate. The LDH Activity Assay kit (Sigma-Aldrich, Saint Louis, MO, USA) is based in the reduction of nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide plus hydrogen (NADH), which is specifically detected by colorimetric (450 nm) assay.

Statistical Analysis.

Statistical analyses were performed using IBM SPSS Statistics software version 21, (New York, USA). Results are reported as mean values ± standard deviation (SD) of samples collected during the first void, unless stated otherwise. Kolmogorov-Smirnov test was used to check for normality of cumulative data distribution. Unpaired Student's *t*-test with Welch's correction and Mann-Whitney U-test were used for statistical analysis between groups when parametric or nonparametric data was considered, respectively. For multiple comparisons, one-way ANOVA nonparametric Kruskal-Wallis test with Dunn's post test modification was used. Correlation between variables was analyzed using Pearson or Spearman test according to the normality of data distribution. $P<0.05$ (two-tailed) values were considered statistically significant. The diagnostic potential of urinary ATP measurements was assessed by receiver operating characteristics (ROC) plots. Area Under the Curve (AUC) comparisons were made using Hanley and McNeil method with MedCalc software (Ostend, Belgium) (Hanley & McNeil, 1983).

RESULTS

Table 2 shows that urinary ATP concentration was significantly ($p<0.001$) higher in BOO patients than in asymptomatic controls. This was also verified when urinary ATP was normalized to urine creatinine levels (ATP/Cr, pmol/mg, $p<0.0001$) to discard a putative kidney influence on urinary ATP (Table 2, see Silva-Ramos et al., 2013b). It is worth to mention that urine creatinine levels were not significantly different ($p>0.05$) between the two studied groups (Table 2). Damage of cells does not account to the higher ATP concentrations found in the urine of BOO patients, because urinary LDH activity was consistently low, did not significantly differ between the two groups (1.80±0.63 U/ml in BOO patients vs.

1.63±0.17 U/ml in asymptomatic controls; p>0.05) and no correlation was observed between urinary ATP values and LDH activity in BOO patients (r=-0.085, p=0.467) (Table 3).

The ROC graphs are commonly used in medical decision making to depict true positive vs. false positive rates. A high AUC is consistent with urinary ATP concentration as such (0.91; 95% CI 0.86 to 0.96; p<0.001) or adjusted to urine creatinine levels (0.88; 95%CI 0.81 to 0.94; p<0.001) being high-sensitive non-invasive biomarkers for discriminating symptomatic BOO patients from asymptomatic controls (Figure 15). There were no significant (p>0.05) differences between AUCs of ATP and ATP/Cr. By selecting the cutoff point of 1.36 nM for ATP and 1.31 pmol/mg for ATP/Cr using Youden's index (Youden, 1950), the sensitivity and specificity were 85% and 80% respectively for ATP (nM) and 80% and 80% respectively for ATP/Cr.

Taking into consideration that BOO patients voided smaller (p<0.001) volumes of urine compared to control individuals (see Table 2) we corrected urinary ATP by the corresponding voided volume. After this normalization, urinary ATP amounts (expressed in nmol per void) were still significantly (p<0.0001) higher in BOO patients than in asymptomatic controls (Table 2).

Table 2 - Urinary measurements in BOO patients and in assymptomatic controls.

	Controls n=22 Mean±SD	**BOO** n=79 Mean±SD	*p* value
ATP (nM)	1.03±0.18	2.62±0.19	**<0.001**
Voided volume (ml)	265.80±24.69	177.10±9.62	**<0.001**
ATP x Voided volume (nmol)	208.80±34.62	456.00±36.00	**<0.0001**
ATP/Cr (nmol/mg)	1.11±3.02	3.99±4.80	**<0.0001**
Creatinine (mg/dl)	116.20±10.20	96.70±5.77	ns
LDH (U/ml)	1.80±0.63	1.63±0.17	ns

Moreover, data from Figure 16 suggest that there is a positive correlation between the ATP amount in urine samples and voided volumes both in asymptomatic men (r=0.584, p<0.005, n=22) and in BOO patients (r=0.5170, p=0.0001, n=79). As a matter of fact, the slope of the interpolation line between urinary ATP and voided volumes was steeper in BOO patients (2.095) than in asymptomatic controls (0.837) (Figure 16), indicating that the urine of BOO patients possesses higher average ATP amounts than control individuals when the samples were corrected for the voided volume.

Table 3- Characteristics of patients with BOO and their correlation to urinary ATP (nmol). *p* values refer to the significance of the correlation.

	Mean ±SD	Pearson's *r*	*p* value (*n*)
Qmax (ml/s)	9.15±3.01	**-0.310**	**0.005** (76)
Siroky class	-1.76±0.09	**-0.324**	**0.004** (76)
VQImax	0.64±0.03	**-0.320**	**0.012** (50)
PVR (ml)	68.63±7.65	0.097	0.509 (50)
VV (ml)	177.10±9.62	**0.517**	**<0.001** (76)
VV+PVR (ml)	242.40±14.02	**0.556**	**<0.001** (50)
LDH (U/ml)	1.63±0.17	0.085	0.467 (75)
Prostatic Volume (ml)	78.18±4.41	-0.048	0.683 (76)
PSA (ng/ml)	3.32±0.25	-0.033	0.783 (74)
IPSS	15.07±0.73	-0.735	0.269 (76)
Voiding subscore	9.07±0.50	-0.044	0.709 (76)
Storage subscore	6.00±0.40	-0.178	0.124 (76)
QOL score	3.79±0.16	-0.087	0.454 (76)

We also explored possible correlations between urinary ATP amounts with a set of clinical parameters in patients with BOO due to BPH (Table 3). BPH patients included in this study reported moderate urinary symptoms (average

IPSS score of 15.3±6.6 from a maximum of 35 ponts,n=79) *i.e.* well above the bothersome LUTS defined as an IPSS score over 7 (see Table 3). None of the parameters recommended for clinical assessment of patients suffering from prostatism correlated positively with urinary ATP. More importantly, we found an inverse correlation between maximal uroflow rates (Qmax, ml/s) measured during voiding at normal desire and urinary ATP amounts in patients with BOO due to BPH (Table 3). Uroflow rate is highly dependent on the voided volume (VV) and flow-volume relationships (e.g. Siroky normogram) are commonly used, although with certain limitations, in the clinical practice (see e.g. Agarwal et al., 2014).

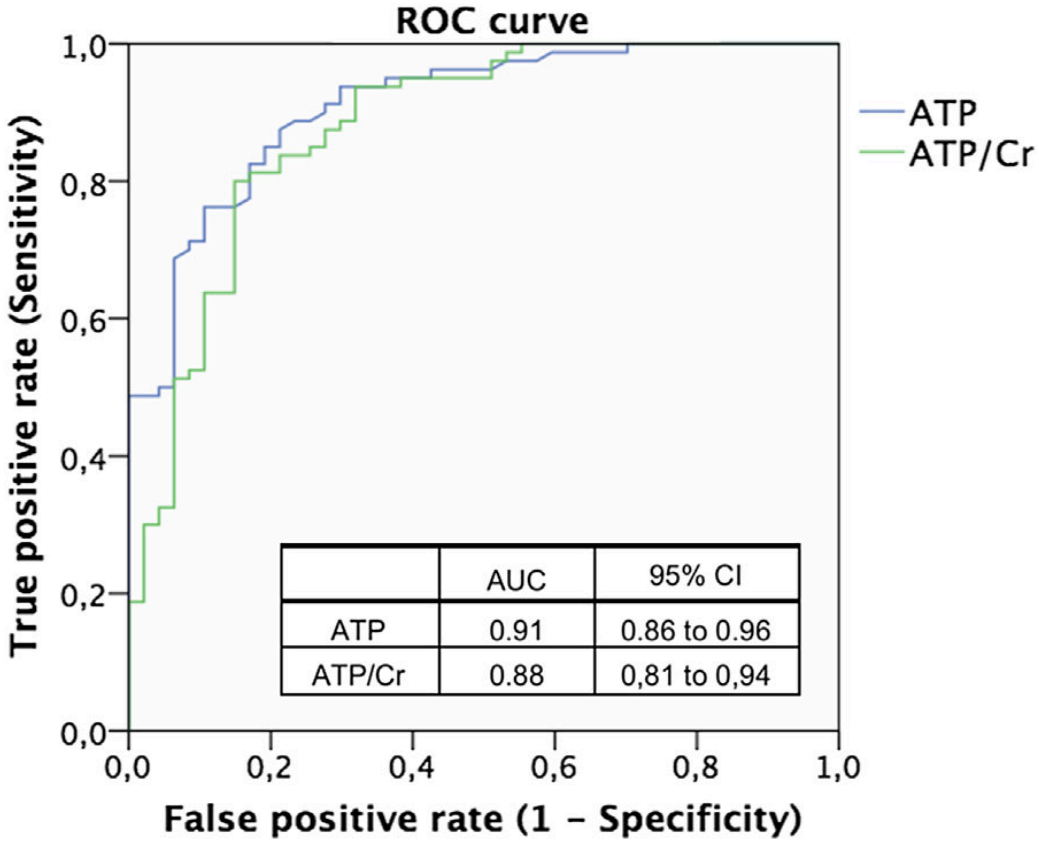

Figure 15 – Receiver-Operator Characteristics (ROC) curves of urinary ATP concentration as such (ATP, blue line) and corrected for the urinary creatinine (ATP/Cr, green line) plotted as continuous variables to discriminate true positive from false positive rates among BOO patients and asymptomatic controls. An area under the curve (AUC) value of 1.0 indicates perfect discrimination value without any overlap, whereas a value of 0.5 indicates a matter of chance. Please note that both ATP and ATP/Cr have AUCs significantly ($p<0.001$) superior to 0.5 (null hypothesis), but there were no differences ($p>0.05$) between the two variables.

The European Association of Urology guidelines also recommend documentation of PVR because bladder volume (VV+PVR) may have a higher

discriminatory value in interpreting flow rates and this parameter may indicate the degree of impact of BOO on detrusor function in uroflow reports. Data in Figure 17 show that urinary ATP reached maximal values in BOO patients with flow-volume relationship values three standard deviations below the average according to the Siroky normogram (Siroky et al., 1979) (see also Table 3). Both voided volume (VV, r=-0.517, p<0.001) and bladder volume (VV+PVR, r=-0.556, p<0.001), but not the post-void residual urine (PVR, r=-0.097, p=0.509), positively correlated with urinary ATP (Table 5). We also found a negative correlation between urinary ATP amounts and the volume-normalized uroflow rate index (VQImax = Qmax/ $\sqrt{(VV + PVR)}$ (r=-0.267, p=0.032) (Agarwal et al., 2014).

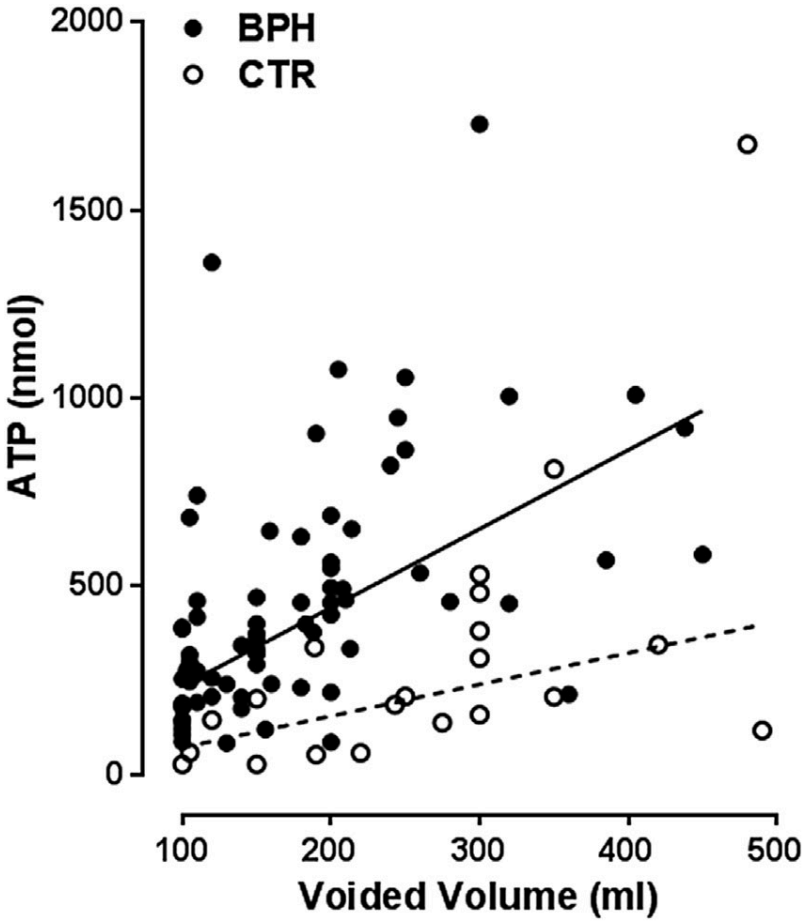

Figure 16 -Relationship between urinary ATP amounts per void (nmol) and voided volumes (ml) in BOO patients (black circles) and asymptomatic controls (white circles). Please note that the slope of the interpolation line between urinary ATP and voided volumes was steeper in BOO patients (full line, 2.095) than in asymptomatic controls (dotted line, 0.837).

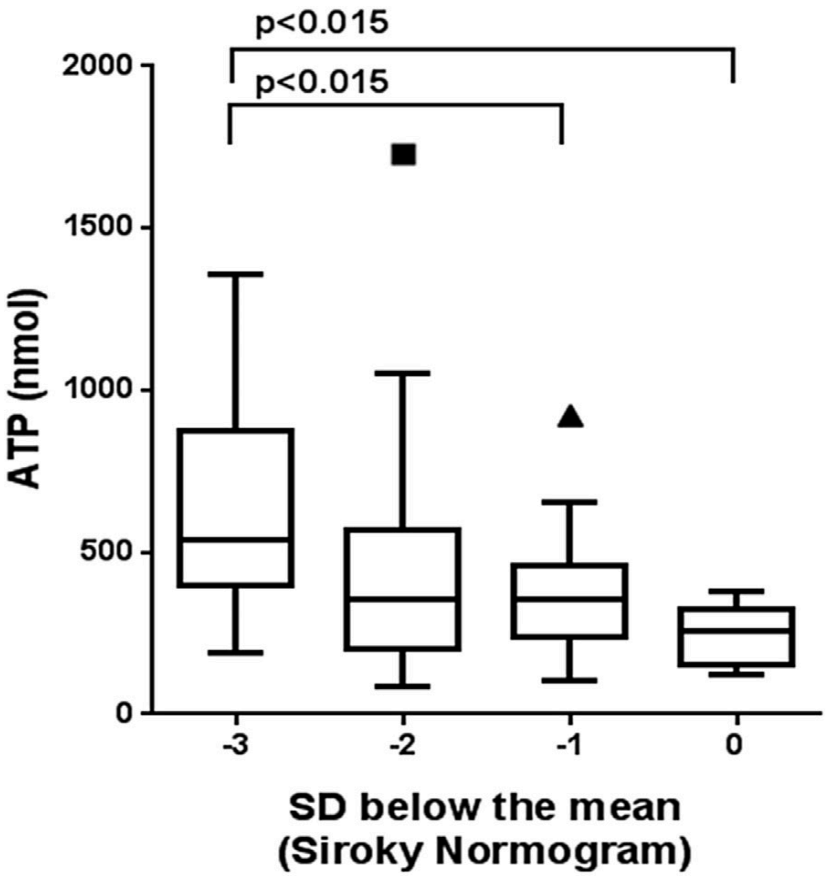

Figure 17 - Relationship between urinary ATP amount per void (nmol) and the Qmax. Abscissa values represent the standard deviation below the mean for Qmax using the Siroky nomogram. Patients with lower Qmax (3 SD below the mean) had significantly higher urinary ATP (p=0.015, one-way ANOVA). Whiskers and outliers (squares and triangles) are plotted according to Tukey's method.

Significant correlations between urinary ATP and uroflowmetry tests contrast with often reported lack of correlation among Qmax and patients perception of BOO-related urinary symptoms (IPSS score, r=-0.039, p=0.735) and/or dissatisfaction concerning their quality of life (QOL score, r=-0.087, p=0.454), respectively. Nonetheless, 18 out of 79 BOO patients exhibiting high ATP per void (>400 nmol, above the median) and low uroflow rate (Qmax < 10 ml/s) had significantly higher IPSS scores than the rest of the population (mean difference of 4.04±0.89, p<0.001). This feature may be mostly attributed to increases in urinary voiding symptoms compared to urinary storage symptoms; a mean difference of 4.50±0.11 (p<0.001, n=18) was observed between the IPSS voiding and storage subscores, but such a difference did not exist in the group of patients with low ATP amounts per void and higher flow rates (mean difference of 2.44±0.82, p>0.05, n=59).

DISCUSSION

Here we show that urinary ATP is significantly higher in patients with BOO due to BPH than in asymptomatic controls. Although it is difficult to prove that urinary ATP obtained by voiding at normal desire is derived mainly from the bladder mucosa, prior investigations have demonstrated that the uroepithelium plays a dominant role (Sui et al., 2014). Since the pioneering report by Ferguson et al. (Ferguson et al., 1997), mounting evidences support the role of mechanical stress (pressure, stretch, hypoosmotic stimulation) as a trigger for ATP release from the urothelium. In fact, ATP can mediate pressure signals in several organ systems, from controlling vascular tone in response to stretch (Bodin & Burnstock, 1996) to transducing touch sensations in the skin (Nakamura & Strittmatter, 1996). So, during normal bladder filling, ATP is released by the urothelium as the bladder distends. The voiding reflex is triggered when urothelial cells deformation by the progressive rise in bladder wall tension reaches a threshold that is proportionate to the internal pressure times half of the radius (Laplace's Law). The higher the bladder volume and, subsequent, wall tension the higher amount of ATP is released from urothelial cells. During voiding an extra pressure is delivered to the urothelium by the underlying smooth muscle contraction. Since the detrusor of patients with BOO due to BPH has to generate more pressure to overcome obstruction it is plausible that more ATP is released into the urine of these patients providing that contractility of the detrusor is maintained or exaggerated. Our data show that the total amount of ATP normalized per voided volume in the urine of BOO patients is much higher than in asymptomatic controls despite the latter voided higher volumes of urine (increased bladder distension), yet, no correlation was found between urinary ATP and the PVR in obstructed patients. Using mucosal strips from the human bladder stimulated electrically to increase the isometric tissue tension, we reported previously that patients with BOO due to BPH release five times more ATP than control individuals (Silva et al., 2015).

Besides the increase in mechanically-induced ATP release from the urothelium of BOO patients, other mechanisms may also account to the higher urinary ATP amounts in these patients. It has been reported that ATP can cause more ATP to be released by urothelial cells creating a "vicious cycle" through the activation of P2 purinoceptors (Sun et al., 2001). Besides this, decreased ATP

catabolism has also been proposed to explain higher amounts of urinary ATP in BOO patients compared to asymptomatic controls. Reports from our and other groups have shown that the ATP catabolism is hindered in the bladder of patients with BOO and detrusor overactivity (Harvey et al., 2002; Silva-Ramos et al., 2015a). Decreases in the activity of ecto-NTPDases have been observed both in the mucosa (unpublished observations from our group) and in the smooth muscle layer, where ATP released from parasympathetic nerves can reach higher amounts in BPH patients than in controls (Silva-Ramos et al., 2015a). Whether surplus ATP accumulation in the detrusor has impact on ATP concentrations being measured in the urine of BPH patients is a matter of debate and requires experimental confirmation. We can, of course, speculate whether the urothelial barrier is damaged on the bladder of BPH patients, allowing more ATP from the muscle and nerves to be detected in the bladder lumen, yet evidence for this assumption is still lacking. Nonetheless, strengthening the purinergic tone through the abnormal production, release and metabolism of ATP is possible in obstructed bladders, as it has been increasingly reported in other urological diseases (reviewed in Burnstock, 2011).

In a previous study, Sugaya and co-workers proposed that measurement of urinary ATP can be used as a marker of pathologic bladder function (Sugaya et al., 2009). Our group added some information suggesting that urinary ATP may be a highly sensitive dynamic biomarker for assessing detrusor overactivity in women with OAB syndrome (Silva-Ramos et al., 2013b). Although it is estimated that significant number of BPH patients also have detrusor overactivity (Andersen & Nordling, 1980), we could not control this variable due to ethical constrains limiting the use of unnecessary invasive urodynamic tests (e.g. filling cystometry and pressure flow studies) in patients with moderate LUTS like the group analyzed in this study. The European Association of Urology guidelines consider invasive urodynamic tests as optional, unless more accurate diagnostic is required in severe cases of LUTS in young individuals or in patients with normal urinary flow rates, when neurologic dysfunction is suspected, and in patients previously submitted to prostatic surgery (Gratzke et al., 2015). None of such cases were included in our study. In the Sugaya's study, ATP/Cr levels in the urine of BPH patients decreased 4 weeks after blockage of α_1-adrenoceptors with tamsulosin along with the improvement of LUTS, thus suggesting that alleviating

outlet resistance may reduce urinary ATP concentrations (Sugaya et al., 2009). The lack of correlation between urinary ATP and prostate size, PSA values and post-void residual urine observed in our study (see also (Sugaya et al., 2009) contrasts with the significant inverse correlation between urinary ATP and uroflowmetry parameters, namely maximal flow rate (Qmax), volume-normalized flow rate index and flow-volume Siroky categories, thus suggesting that urinary ATP amounts reflect most probably the activity of the bladder above the obstruction. Correlation between bladder activity and urinary ATP levels is perhaps more straightforward in women with overactive bladders as they do not have a prostate gland, but the argument may be still valid in men's bladders.

Looking at the relationship between urinary ATP and patients' perception about LUTS severity, we could not find any significant correlation when the total IPSS or IPSS subscores were used in the entire population of BPH patients. This is not surprising since, the IPSS does not correlate with or predict urodinamically documented BOO and the same also applies to prostate size, PSA values and post-void residual urine (Yoshida et al., 2004). It is also known that non-invasive tests to study the dynamics of urine flow are more reliable than subjective symptoms in predicting BOO due to BPH, but artifacts can also appear from poor technique or poor interpretation of the curves obtained. False Qmax values may occur, which can be circumvented by taking into account the shape of the uroflow curve and the voided/bladder volume. As a matter of fact, we show here that urinary ATP correlates inversely with Qmax and also with the flow-volume scores given by the Siroky normogram and the volume-corrected flow rate index. Urinary ATP positively correlates with the urine volume inside the bladder before voiding that can be appreciated summing the VV plus the PVR. As per the Laplace's Law, force-relationship of detrusor contraction depends on luminal pressure, bladder volume and state of muscle activation and is characteristically hyperbolic. Thus, the force and velocity of contraction of detrusor corresponds to the "vis-a-tergo" strain generated by bladder filling, *i.e.* VV+PVR in an uroflow analysis. Taking this into account, Agarwall et al. found that VQImax was more discriminatory than the respective values of Qmax in the investigation of the degree of bladder dysfunction in patients with BOO, neurogenic bladder and detrusor underactivity (Agarwal et al., 2014). Anyway, these results strengthen our assumption that urinary ATP may be an important marker of urinary bladder tension rising

proportionately to urine accumulation (bladder radius) and to detrusor contraction (pressure) in order to overcome bladder outlet resistance during voiding. Therefore, in BPH patients with low flow rates urinary ATP per void may discriminate positively increases in urethral resistance (bladder wall tension due to obstruction) from patients exhibiting detrusor hypoactivity, as the latter may present low urinary ATP amounts. As a matter of fact, we found that the discriminatory power of urinary ATP increased significantly in BOO patients with a Qmax < 10 ml/s and in those with a Qmax below the -1 SD line in the flow-volume Siroky normogram when a lower end cut-off amount of ATP of 400 nmol is imposed. Using these criteria, we found that voiding symptoms perceived by BPH patients gained significance compared to the IPSS storage subscore in patients presenting higher ATP per void and lower flow rates. It should, however, be emphasized that a superior value obtained by increasing the number of evaluated parameters does not underscore the importance of urinary ATP normalized by the voided volume or by the urinary creatinine which also have high AUCs on ROC graphs (~0.90).

Even though we have recruited BOO patients and asymptomatic controls with the same age range (40-79 years of age), the mean age in both groups reflects the epidemiology of BPH disease which tends to affect older men (66±9 years of age in BOO patients vs. 53±11 years of age in asymptomatic controls). Controversy still exists in the literature concerning the relevance of age in urinary ATP amounts; there is one paper showing that ATP release increases with age (Yoshida et al., 2004), yet others found no relationship between utinary ATP and age (Gill et al., 2015; Sugaya et al., 2009). In our study, we also failed to find any significant correlation between age and urinary ATP in the control group (r=-0.051, p=0.412). This was also verified extending the age range of asymptomatic controls to younger individuals (20-83 years of age, mean 42±15 years; r=0.067, p=0.629, n=48). Covariance analysis of our data showed that the higher urinary ATP amounts in BOO patients is independent of age (p<0.001). Under these circumstances, it is not plausible that age alone account significantly to the observed differences in urinary ATP between asymptomatic controls and BOO patients.

It has been shown in previous studies that urinary ATP positively correlates with bladder distension by saline instillation during cystometry testing

(Cheng et al., 2014), as well as with the voided volume in healthy women (Silva-Ramos et al., 2013b). Data presented here show that the same also occurs in asymptomatic men. This is in keeping with the concept that urinary ATP originates mostly from the physiological distension of the urothelium (Ferguson et al., 1997; Wang et al., 2005). While a similar situation occurs in BOO patients, the slope of the interpolation line between the total urinary ATP normalized per voiding volume was significantly steeper than that in asymptomatic controls. This normalization was necessary because the voiding volumes obtained from BPH patients were smaller than those verified in the controls and we wanted to compare the amount of ATP released into the bladder lumen in isovolumetric conditions. Therefore, one may conclude without much hesitations that the urinary bladder of BOO patients release higher amounts of ATP during voiding at normal desire, confirming our *in vitro* experiments using surgical samples from the mucosa of some of these patients stimulated electrically (Silva et al., 2015). As per the Laplace's law, keeping the radius (urine volume) constant the increase in urinary bladder tension and, thus, ATP release, essentially depends on detrusor contraction (pressure) to overcome outlet resistance during voiding.

Increased ATP release from uroepithelial cells resulting in urinary accumulation of the nucleotide has also been observed in patients with OAB presenting detrusor overactivity (Kumar et al., 2010; Silva-Ramos et al., 2013b) and painful bladder syndrome (Kumar et al., 2007; Sun et al., 2001). It is worth to mention that these two conditions are complex clinical syndromes without any identifiable cause that affect mainly women and are based on self-reported symptoms of urinary urgency and frequency, associated or not with incontinence in the first situation and chronic pelvic pain or discomfort in the latter. In the present study, we used male patients selected for prostatic surgery to relieve BOO due to BPH. Notwithstanding this, a significant number of BPH patients may present detrusor overactivity (Andersen & Nordling, 1980), yet in this case diagnostic accuracy might increase if invasive urodynamic testing for involuntary contractions of the detrusor muscle during bladder filling is performed in addition to non-invasive uroflowmetry combined with urinary biomarkers, such as ATP. Gender differences must also be taken into account when comparing urinary ATP concentrations in any clinical condition; this is because for yet unknown reasons, we and others found higher urinary ATP concentrations in healthy women

compared to age-matched men (2.65±0.40 nM, n=36 vs. 1.03±0.18 nM, n=22, respectively; p<0.001) (cf. Silva-Ramos et al., 2013b; Sugaya et al., 2009). Besides gender preference and chronic pelvic pain, increased urinary ATP concentrations observed in patients with bladder pain syndrome / interstitial cystitis would be best valorized if combined with other potential biomarkers targeting alterations of the urothelium barrier function that are characteristic of this condition; these include glycoprotein antiproliferative factor (APF), epidermal growth factor (EGF), and heparin-binding EGF-like growth factor, among others (Fry et al., 2014).

In most publications, urinary biomarkers are normalized to the urinary concentration of creatinine in order to reduce sample variation and to discard kidney influence on ATP amounts. Here, we found that the urinary creatinine was about the same (p>0.05) in BOO patients and asymptomatic controls. Our data show that the predictive value of urinary ATP to determine the existence of increased bladder pressure due to outlet obstruction is roughly similar either adjusting or not ATP amounts to the urinary concentration of creatinine. Overall these findings demonstrate that urinary creatinine concentration does not accompany urinary ATP accumulation and further strengths our argument that most urinary ATP originates in the lower urinary tract particularly in the bladder and not upstream in the kidney. Under these conditions, adjusting ATP amounts to urinary creatinine is meaningless.

We are also aware that to validate a diagnostic biomarker one should compare its performance to the gold standard method, which is in this case the urodynamic pressure flow study, in order to document increased detrusor pressure and decreased urinary flow. Notwithstanding this, while we could measure urinary ATP amounts in asymptomatic controls, it would not be ethical to perform invasive urodynamic tests in this population. This constrain would also limit the comparative analysis of specificity / sensitivity of both methods using ROC graphs. Anyway these results support a role of ATP as a possible marker of obstruction and should encourage further studies examining the relationship between urinary ATP and specific urodynamic tests (e.g. pressure flow study) in more severe cases of BOO.

Another limitation for the use of urinary ATP measurements in clinical practice implies the meticulous exclusion of patients with urinary tract infections

and malignancy. For decades urinary ATP as been proposed as a marker of bacterial infection of the urinary tract (Thore et al., 1975). Although never used in clinical practice, this concept has recently been disputed since urine samples from patients with positive urine culture had lower ATP concentrations than controls (Gill et al., 2015), which might happen because inflammatory cells release, but also metabolize extensively, the nucleotide by intracellular and membrane-bound nucleotidases. Anyway, we excluded urinary tract infections by urine culture and a significant intracellular source of ATP due to breakdown of urothelial cells by detecting low LDH activity in tested samples.

In conclusion, the results obtained in this study support an important role of ATP in the pathophysiology of LUTS due to BPH. Furthermore, the putative role of urinary ATP as a dynamic biomarker of BOO should also be considered. In fact, a high area under the ROC curve is consistent with urinary ATP either alone or adjusted to urinary creatinine being a highly-sensitive pressure transducer biomarker for discriminating detrusor competence when comparing BOO patients with low urinary flow rates. AUC scores of about 90% are similar to predictive urine test strips for diagnosing urinary tract infections (Sultana et al., 2001) and are significantly higher than the PSA value for diagnosing prostate cancer, which is around 70% (Thompson et al., 2005). The possibility of diagnosing BOO with a non-invasive biochemical test is appealing. While BPH is a very common disorder, the diagnostic of associated BOO is difficult in the clinical practice. Overall LUTS are poor predictors of BOO (de la Rosette et al., 1998) and symptoms alone are insufficient for the diagnosis. Most of the time, the diagnostic of BOO is inferred from symptoms and signs, ultrasound examination of the lower urinary tract and uroflowmetry testing. None of these assessments are individually very accurate, but altogether portray a fairly good idea of the clinical status of the patient in terms of BOO. In more doubtful situations, which are not that uncommon, more invasive exams like pressure-flow study are needed. Moreover, one condition that is getting more and more attention which troubles the clinical diagnostic of BOO is voiding dysfunction characterized by low flow rates due to detrusor underactivity. This clinical entity is very common in aged patients and its prevalence seems to be rising (Osman et al., 2014). Until now the only exam able to distinguish detrusor underactivity from BOO is the pressure flow study. There is, therefore, an unmet clinical need for a simple, non-

invasive test to screen these patients. Although this study did not address specifically this issue, we may speculate about the discriminative power of urinary ATP per void in BOO patients presenting detrusor hypoactivity. According to our prediction, low urinary ATP amounts per void are expected to occur in these patients due to failure in increasing the bladder pressure and, thus, the nucleotide release from the urothelium, as a consequence of obstruction.

ACKNOWLEDGEMENTS

This research was partially supported by Fundação para a Ciência e a Tecnologia (FCT, FEDER funding, projects PTDC/SAU-OSM/104369/2008 and PEst-OE/SAU/UI0215/2014) and Associação Portuguesa de Urologia (APU). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Isabel Silva was in receipt of a PhD Studentship from FCT (SFRH/BD/88855/2012). The authors wish to thank Mrs. Helena Costa e Silva and Belmira Silva for their valuable technical assistance.

STATEMENT OF CONFLICTS OF INTEREST

None

ARTICLE 4

URINARY ATP MAY BE A DYNAMIC BIOMARKER OF DETRUSOR OVERACTIVITY IN WOMEN WITH OVERACTIVE BLADDER SYNDROME

Miguel Silva-Ramos[1,2#], Isabel Silva[1#], Olga Oliveira[1,2], Sónia Ferreira[1,2], Maria Júlia Reis[3], José Carlos Oliveira[3], Paulo Correia-de-Sá[1*]

[1]Laboratório de Farmacologia e Neurobiologia, UMIB, Instituto de Ciências Biomédicas Abel Salazar (ICBAS) - Universidade do Porto (UP), Porto, Portugal,
[2]Serviço de Urologia - Centro Hospitalar do Porto (CHP), Porto, Portugal, [3]Serviço de Química Clínica - Centro Hospitalar do Porto (CHP), Porto, Portugal

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[#]MSR and IS contributed equally to this work

Author contribution:
Miguel Silva-Ramos: conception and design of the study, patients selection and recruitment, analysis and interpretation of data, statistical analysis, drafting of the manuscript.
Isabel Silva: samples processing/preservation, ATP, NGF and LDH determinations, analysis and interpretation of data, statistical analysis
Olga Oliveira: samples processing/preservation, acquisition, analysis and interpretation of data, statistical analysis.
Sónia Ferreira: samples processing/preservation, acquisition, analysis and interpretation of data.
Maria Julia Reis: biochemistry and urine analysis.
José Carlos Oliveira: biochemistry and urine analysis, NGF quantification.
Paulo Correia-de-Sá: conception and design of the study, project supervision, analysis and interpretation of experimental data, statistical analysis, revision of the manuscript.

ABSTRACT

Background: Nowadays, there is a considerable bulk of evidence showing that ATP has a prominent role in the regulation of human urinary bladder function and in the pathophysiology of detrusor overactivity. ATP mediates nonadrenergic-noncholinergic detrusor contractions in overactive bladders. In vitro studies have demonstrated that uroepithelial cells and cholinergic nerves from overactive human bladder samples release more ATP than controls. Here, we compared the urinary ATP concentration in samples collected non-invasively from OAB women with detrusor overactivity and age- matched controls.

Methods: Patients with neurologic diseases, history of malignancy, urinary tract infections or renal impairment (creatinine clearance <70 ml/min) were excluded. All patients completed a 3-day voiding diary, a 24 h urine collection and blood sampling to evaluate creatinine clearance. Urine samples collected during voluntary voids were immediately freeze-preserved for ATP determination by the luciferin-luciferase bioluminescence assay; for comparison purposes, samples were also tested for urinary NGF by ELISA.

Results: The urinary content of ATP, but not of NGF, normalized to patients' urine creatinine levels (ATP/Cr) or urinary volume (ATP.Vol) were significantly ($P<0.05$) higher in OAB women with detrusor overactivity (n=34) than in healthy controls (n = 30). Significant differences between the two groups were still observed by boosting urinary ATP/Cr content after water intake, but these were not detected for NGF/Cr. In OAB patients, urinary ATP/Cr levels correlated inversely with mean voided volumes determined in a 3-day voiding diary.

Conclusion: A high area under the receiver operator characteristics (ROC) curve (0.741; 95% CI 0.62–0.86; $P<0.001$) is consistent with urinary ATP/Cr being a highly sensitive dynamic biomarker for assessing detrusor overactivity in women with OAB syndrome.

INTRODUCTION

Overactive bladder is a complex clinical syndrome based on self-reported symptoms of urinary urgency associated with incontinence in up to one-third of cases, usually accompanied by daytime frequency and nocturia, in the absence of proven infection or other obvious pathology (Abrams et al., 2002). It has been reported an overall prevalence of 12.8% in women (Irwin et al., 2006), and a significant impact on patients quality of life (Coyne et al., 2011). The precise pathogenesis underlying OAB remains to be clarified and might be multifactorial. OAB symptoms are often associated with detrusor overactivity, which is diagnosed by invasive urodynamic testing as involuntary contractions of the detrusor muscle during bladder filling. Regrettably, the relationship between clinical symptoms and urodynamic findings is not reliable (Bates et al., 1970; Hashim & Abrams, 2006). In fact, procedure constrains such as saline infusion rate and temperature, patient position, and anxiety, may yield distinct urodynamic results (Brostrom et al., 2002), which makes urodynamic confirmation of detrusor overactivity with only limited clinical value regarding the severity or prognosis of idiopathic OAB (Nitti et al., 2010; Rovner et al., 2011). This renders urodynamic investigation with suboptimal characteristics to evaluate OAB patients and urge the search for new accurate, reliable and non-invasive tests to predict detrusor overactivity and to assess patients' therapeutic outcome (reviewed in Farag & Heesakkers, 2011).

Recently, low grade inflammatory mediators, such as cytokines, prostaglandin E_2 (PGE_2) and NGF, gained significant attention as urinary biomarkers of detrusor overactivity. Although they correlate with OAB symptom severity, they have not been shown to have independent prognostic benefit (reviewed in Cartwright et al., 2011) and they still cannot replace the standard filling cystometry in standard clinical practice (Farag & Heesakkers, 2011). For instance, pilot studies indicate that there is a wide variation of urinary NGF levels and not all patients with OAB have elevated urinary NGF (Kuo et al., 2010; Liu et al., 2010; Ochodnicky et al., 2011).

Purinergic transmission has increasingly been accepted as having an important role in urinary tract dysfunction. It encompasses both efferent and afferent paths of the voiding reflex, and has particular relevance in the pathogenesis of detrusor overactivity (Burnstock, 2011; Ruggieri, 2006). Several

studies have reported that urothelium releases ATP in response to mechanical and chemical stimuli (Ferguson et al., 1997; Wang et al., 2005). These stimuli evoke a discharge in suburothelial low-threshold sensory nerves through P2X3 receptors activation, since sensory nerve excitation is substantially reduced by P2X3 antagonists (Ito et al., 2008) and in P2X3 knock-out mice (Cockayne et al., 2000), also the density of these receptors is increased in patients with detrusor overactivity (Moore et al., 1992). Conversely, intravesical instillation of ATP increases bladder activity in a concentration dependent manner (Pandita & Andersson, 2002); intravesical ATP decreases bladder capacity and micturition volume, with limited effect on detrusor pressure. Recently, evidence has been emerging in support of a role for suburothelial myofibroblasts in detrusor contraction, which also express P2X and P2Y purinoceptors (Fry et al., 2007). In detrusor smooth muscle fibres, P2X1 receptor subtype expression is also markedly increased in unstable bladders (reviewed in Burnstock, 2011). This implies that ATP is an important mediator of bladder sensory/effector pathways.

Increased ATP release from the urothelium has also been demonstrated in animal models of OAB (Khera et al., 2004; Smith et al., 2005). *In vitro* studies conducted in our lab, as well as by others, showed that uroepithelial cells and cholinergic nerves from overactive or obstructed human bladder samples release more ATP than controls (Kumar et al., 2010; Silva-Ramos et al., 2010). Interestingly, strong evidences suggest that the primary source of ATP in the human bladder is the urothelium (Kumar et al., 2004). Thus, this study was prospectively designed to measure the urinary concentration of ATP in OAB women tested positively for detrusor overactivity and in age-matched controls to assess the putative role of ATP as a non-invasive biomarker of detrusor overactivity in OAB patients. For comparison purposes, urine samples were also tested for NGF levels.

METHODS

Patients and procedures

This study and all its procedures were approved by the Ethics Committees of Centro Hospitalar do Porto (CHP) and of Instituto de Ciências Biomédicas de Abel Salazar (Medical School) of the University of Porto (ICBAS-UP). All patients signed an informed consent prior to examinations and for using the biological

material. The investigation conforms to the principles outlined in the Declaration of Helsinki. A total of 70 women were enrolled in this study, including 34 patients with overactive bladder symptoms and detrusor overactivity (aged 28-84 years, mean 57±13 years) and 36 controls (aged 34-67 years, mean 52±9 years) that included asymptomatic volunteers recruited among the hospital staff and patients with mild stress incontinence without overactive bladder symptoms and normal urodynamics (see Figure 18). This was a prospective study. Patients were consecutively selected (January 2010 - June 2011) from outpatients consulting the Department of Urology of CHP complaining from urgency (at least one episode daily), frequency (>8 voids/day) and reduced bladder capacity (mean voided volume <300ml). Detrusor overactivity was confirmed in all women with OAB by urodynamic testing performed within 12 months before collection of biological samples and the results were analysed in accordance to the criteria of the International Continence Society (ICS) defining detrusor overactivity as involuntary and spontaneous contractions of the detrusor occurring during the storage phase. Patients with history of malignancy, pelvic radiotherapy, neurologic disease, any systemic or inflammatory condition, active urinary tract infections, renal impairment (creatinine clearance <70 ml/min) or taking any medication that could affect bladder function within the 14 days time period before sample collection were excluded; pregnant women were also excluded from the study. Patients with incomplete emptying (post-void residual volume >100 ml) and with voiding dysfunction (Pdet at maximal flow >60 mm H_2O) were also not included in the study. All patients completed a 3-day voiding diary, a 24h urine collection and blood sample collection to estimate creatinine clearance. Subjects were asked to void at normal desire into a sterile cup. Thus, urine sample collection in this work was non-invasive, which contrasts with previous studies that used urodynamic fluid voided after cystometric saline bladder filling (Cheng et al., 2013; Cheng et al., 2010). Two consecutive voids were used; the first void was collected upon patients' arrival to consultation and the second void was collected after allowing patients to drink 500 mL of water ad libitum. Voided volume was recorded and post-void residual volume was estimated by ultrasonography. Mid-stream urine samples were divided into three tubes; one for sediment examination, another for creatinine measurement and a third was immediately freeze-dried in liquid nitrogen and preserved at -80ºC until ATP

determination. ATP values were normalized according to urinary creatinine (Cr) values to reduce sample variation and to discard kidney influence on ATP levels. ATP/Cr ratios were compared between patients and controls.

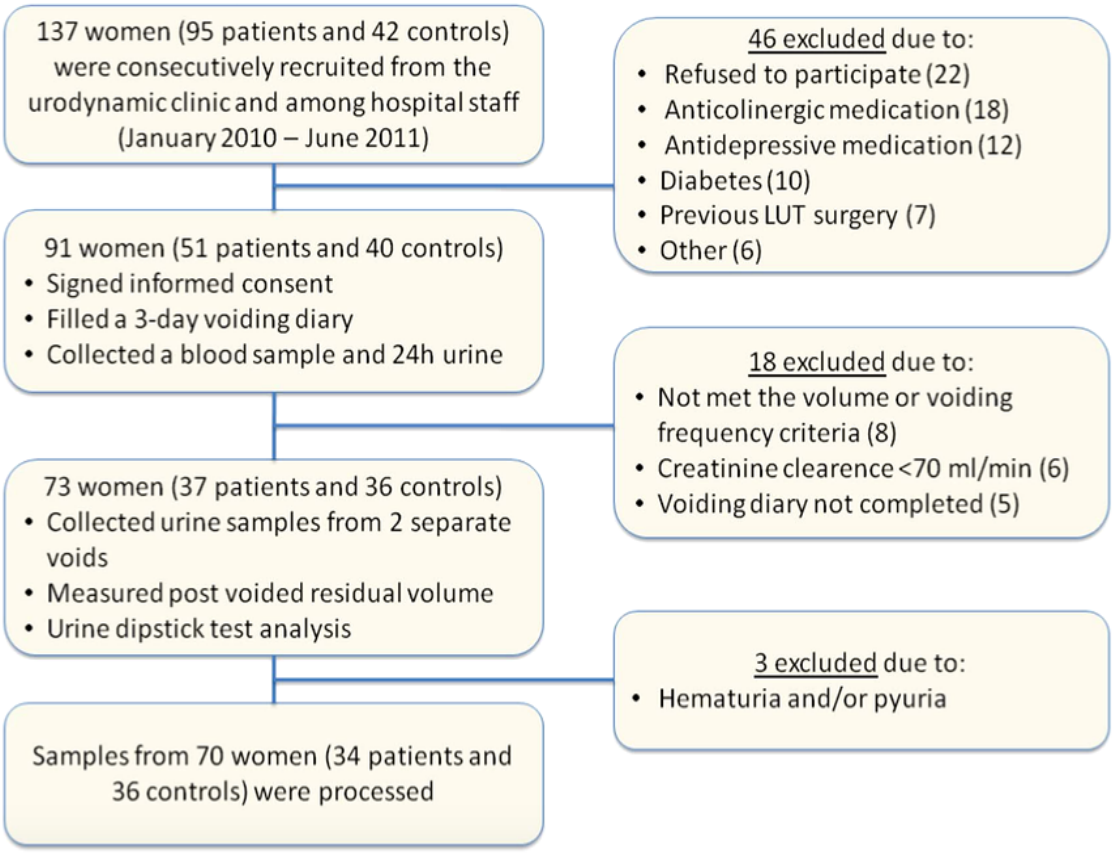

Figure 18. Flow diagram of patient selection

Measurement of urinary creatinine

The quantitative determination of urinary creatinine was performed *in vitro* on a Cobas Integra 800 analyser using the kinetic colorimetric Creatinine Jaffé Gen.2 assay according to the manufacturers' instructions (Roche Diagnostics GmbH, Mannheim, Germany).

Measurement of urinary ATP

Undiluted urine samples were defrosted till 25ºC and afterwards centrifuged at 3000 g at room temperature for 20 seconds to remove cellular debris. The supernatant was separated. A mixture of luciferin-luciferase was added according to the manufacturer instructions using the ATP Bioluminescence Assay Kit HS II (Roche Applied Science Indianapolis, Indiana, USA). ATP detection was evaluated using amulti-mode microplate reader

(Synergy HT, BioTek Instruments Inc., Vermont, USA) controlled via BioTek's Gen5™ Data Analysis Software. Sample bioluminescence was compared to that of standard amounts of ATP used in the same concentration range; standard ATP samples were prepared daily. All samples were run in duplicate. The minimum ATP detection limit in these experimental conditions was 10^{-12} M (10^{-16} moles in 100 µl samples) and luminescence correlated linearly to ATP concentration till 10^{-6} M. Each urine sample remaining was used to quantify the lactate dehydrogenase (LDH, EC 1.1.1.27) activity (Keiding, 1974). LDH is an intracellular enzyme which is commonly used as an indicator of cell integrity providing that its values are kept at a low level.

Measurement of urinary NGF

Measurement of urinary NGF level was also done in a subset of urine samples used for ATP determination (technique reviewed by Kuo et al., 2010). After defrosting and homogenization, samples were used to measure NGF levels by the ELISA method, according to the manufacturer instructions. We determined urinary NGF concentration using the E_{max} ImmunoAssay System (Promega, Madison, EUA), a specific ELISA kit that has a minimum sensitivity of 7.8 pg/ml. All samples were run in duplicate. NGF values were also normalized according to urinary creatinine (Cr) values to reduce sample variation; obtained NGF/Cr ratios were compared between patients and controls.

Routine measurements of blood and urine samples were performed in a blind manner by independent observers from Serviço de Química Clínica - Centro Hospitalar do Porto. Urinary ATP and NGF contents were tested in blind samples at Laboratório de Farmacologia e Neurobiologia (UMIB/ICBAS-UP). NGF measurements were re-tested and confirmed with the methodology in use at the Department of Urology, Centro Hospitalar de S. João and Faculty of Medicine, University of Porto.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.04 software (La Jolla, USA). Results are reported as mean values ± standard deviation (SD) of samples collected during the first void, unless stated otherwise. Kolmogorov-Smirnov test was used to check for normality of data distribution. Unpaired

Student's *t*-test with Welch's correction and Mann-Whitney U-test were used for statistical analysis between groups when parametric or nonparametric data was considered, respectively. For multiple comparisons, one-way ANOVA nonparametric Kruskal-Wallis test with Dunn's post test modification was used. Correlation between variables were analysed using the Spearman test. $P<0.05$ (two-tailed) values were considered statistically significant.

RESULTS

In this study, efforts have been made to match age among OAB women with detrusor overactivity and the control group ($P>0.05$, see Table 4), since it has been described that urinary ATP may increase in older subjects (Sugaya et al., 2009); see in Table 5 the positive correlation between age and ATP/Cr levels found in our series (Spearman test, r=0.380, $P<0.05$). The two groups were also essentially similar ($P>0.05$) regarding creatinine clearance, urine pH and urinary LDH activity (Table 4). Early reports indicate that females with detrusor overactivity may have significantly lower urinary pH than controls (Moore et al., 1990). This tendency did not reach statistical significance ($P>0.05$) in this series excluding any possible influence the pH might have on urinary ATP and NGF content in control and OAB patients. As expected, maximum and mean voided volumes determined from the 3-day voiding diary were significantly ($P<0.05$) lower in OAB patients than in controls (Table 4).

Urinary ATP levels were significantly ($P<0.05$) higher in OAB women with detrusor overactivity as compared to the control group (Table 4 and Figure 19A). This was also verified when the ATP content was normalized either to urine creatinine levels (ATP/Cr, pmol/mg) (Figure 19C) or to the urinary volume (ATP.Vol, pmol) (Table 4); the latter reflects better the raw amount of ATP accumulated in the bladder during urine storage. Since no significant changes ($P>0.05$) were observed in the activity of the intracellular enzyme, LDH, in urine samples from OAB patients and age-matched controls (Table 4) and no correlation was found between ATP/Cr levels and LDH activity (Table 5), urinary cells damage might not account for changes detected in ATP levels in OAB patients and controls.

The ROC graphs are commonly used in medical decision making. ROC graphs have long been used in signal detection theory to depict the tradeoff

between hit rates and false alarm rates of classifiers (Egan, 1975). A high area under the ROC curve (0.741; 95% CI 0.62 to 0.86; *P*<0.001) is consistent with urinary ATP/Cr being a highly sensitive biomarker for discriminating OAB women with detrusor overactivity (Figure 19E).

TABLE 4 - Characteristics of OAB patients and controls. P<0.05 (unpaired Student's t-test with Welch's correction) represent significant differences from controls.

	Controls (n=36) mean±SD	**OAB (n=34) mean±SD**	**P**
Age (yrs)	51.8±8.7	57.3±12.8	0.055
Creatinine clearance (ml/min)	98.9±9.6	116.7±7.1	0.171
Urinary pH	6.7±0.4	5.9±0.3	0.135
Urinary LDH (U/ml)	12.1±3.7	6.8±1.2	0.174
3-Day Voiding Diary:			
Minimum Void Volume (ml)	86.6±21.3	73.7±23.5	0.263
Maximum Void Volume (ml)	425.0±121.5	326.1±85.3	0.009
Mean Void Volume (ml)	198.6±31.1	159.9±40.2	0.033
1st Void (upon arrival):			
Volume 1st Void (ml)	203.3±56.5	210.8±61.7	0.810
Urinary ATP 1st Void (pM)	2647±403	7004±1228	0.001
Urinary ATP x Vol. 1st Void (pmol)	707.8±139.6	1556.1±290.9	0.012
Urinary ATP/Cr 1st Void (pmol/mg)	7.2±1.7	27.5±8.3	0.022
Urinary NGF 1st Void (pg/ml)	530.5±136.2	467.5±193.0	0.397
Urinary NGF x Vol. 1st Void (ng)	117.9±42.3	67.5±15.7	0.126
Urinary NGF/Cr 1st Void (pg/mg)	64.0±13.6	109.5±29.0	0.162
2nd Void (after water intake):			
Volume of 2nd Void (ml)	310.0±77.5	260.9±66.9	0.181
Urinary ATP 2nd Void (pM)	5700±978	10577±1533	0.006
Urinary ATP x Vol. 2nd Void (pmol)	2003.1±366.4	3008.4±517.5	0.022
Urinary ATP/Cr 2nd Void (pmol/mg)	42.5±7.2	89.1±19.3	0.029
Urinary NGF 2nd Void (pg/ml)	526.0±227.1	296.2±128.4	0.185
Urinary NGF x Vol. 2nd Void (ng)	82.7±24.0	39.2±10.4	(0.045)
Urinary NGF/Cr 2nd Void (pg/mg)	86.0±17.4	121.7±33.8	0.352

Although NGF levels normalized to creatinine content in the urine (NGF/Cr, pg/mg) tended to increase in OAB patients as compared to controls,

this trend did not reach statistical significance (*P*>0.05) (Figure 19D; see also Kim et al., 2006; *cf.* Antunes-Lopes et al., 2011). We found no significant correlation between ATP/Cr and NGF/Cr levels measured in parallel from samples collected from OAB patients (Spearman test, r=0.221, *P*>0.05), despite the two compounds showed some degree of correlation in the control group (Table 5). We did not found statistically significant differences in the concentration of NGF (pg/ml) measured in urine samples from control and OAB patients (Figure 19B) and the urinary NGF accumulation during bladder storage normalized to the urinary volume (NGF.Vol, ng) was even lower in OAB patients than in controls (Table 4). We detected a wide variation (and lack of correlation) of urinary NGF/Cr levels in paired samples collected from control individuals between the 1st and the 2nd void obtained after water intake, which was no better in women with OAB (Figure 20B and 20D, see also Table 5). These results suggest that NGF determination is highly dependent on the urinary volume (Table 4), a situation that has not been reported in previous studies. The statistical analysis presented in Table 4, shows that the mean urinary NGF content normalized to voided volume decreased significantly in OAB patients as compared to healthy controls as a consequence of forcing the desire to void by water intake (2nd void). This finding is in clear contrast to that occurring with urinary ATP; the urinary content of the nucleotide boosted in the 2nd void upon increasing the urinary volume by drinking water *at libitum* (for correlation analysis, see Table 5). It is worth noting that water intake (after 1st void) increases the sensitivity of urinary ATP determinations in OAB patients *versus* controls (Table 4, ROC curve area = 0.769; 95% CI = 0.64-0.90; *P*<0.001).

TABLE 5. **Correlation between variables analysed using the Spearman test.** *P*<0.05 values were considered statistically significant.

	Controls		**OAB Patients**	
	r (Spearman approx.)	**P value**	**r (Spearman approx.)**	**P value**
ATP/Cr vs NGF/Cr	0.378	0.023	0.221	0.118
ATP/Cr (1st vs 2nd voids)	0.774	<0.0001	0.381	0.013
NGF/Cr (1st vs 2nd voids)	-0.109	0.291	0.307	0.046
Age vs ATP/Cr	0.380	0.019	-0.198	0.131
Age vs NGF/Cr	0.217	0.134	-0.115	0.165
LDH vs ATP/Cr	-0.054	0.399	0.249	0.085
LDH vs NGF/Cr	-0.185	0.199	-0.450	0.006

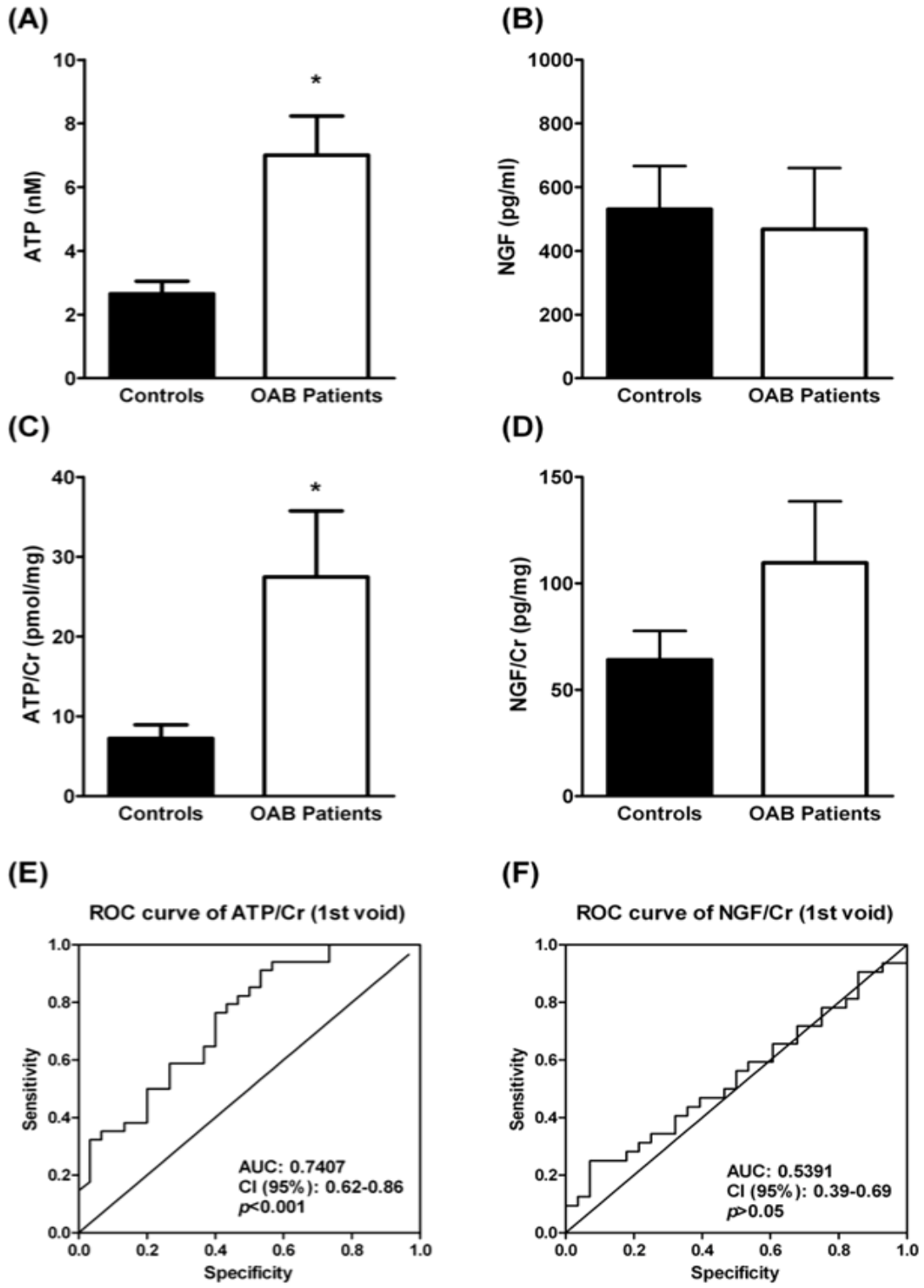

Figure 19. Urinary ATP and NGF levels determined before (A: ATP, nM and B: NGF, pg/ml) and after normalization to urine creatinine content (C: ATP/Cr, pmol/mg and D: NGF, pg/mg) in women with OAB (n = 34) and age-matched controls (n = 36). Columns are mean values ± standard deviation (SD). *P,0.05 (unpaired Student's t-test with Welch's correction) represent significant differences from controls. In E and F, represented are the ROC curves of urinary ATP/Cr and urinary NGF/Cr in OAB patients, respectively. Please note that, for this cohort, ATP/Cr has a better area under de curve (AUC=0.7407; 95% confidence interval (CI)=0.62–0.86; P,0.001) than NGF/Cr (AUC = 0.5391; 95% CI = 0.39–0.69; P,0.001).

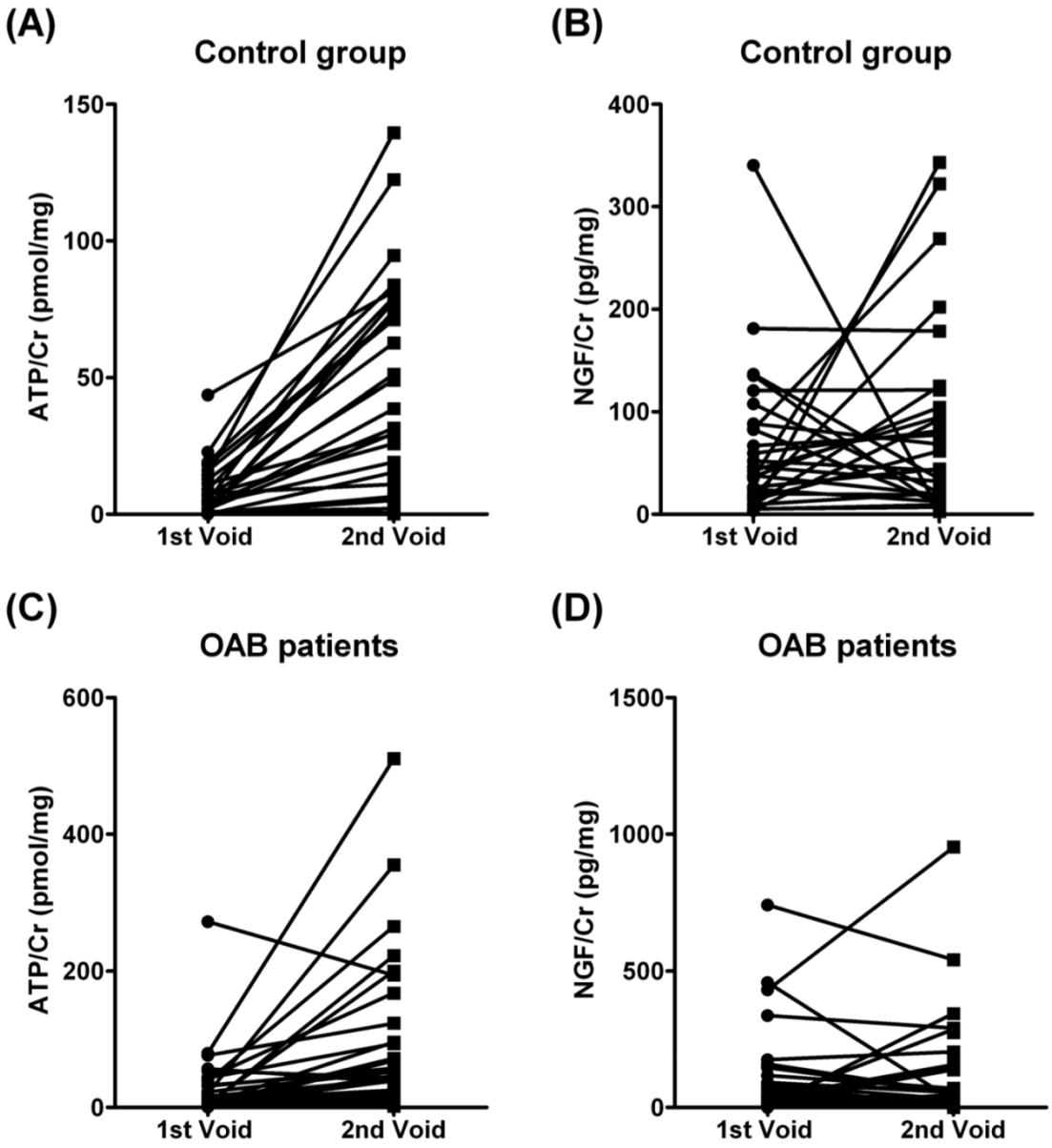

Figure 20. Analysis of ATP/Cr (pmol/mg) and NGF/Cr (pg/mg) content in paired urine samples collected from control women (A and B) and OAB patients (C and D) at arrival to the consultation (1st void) and after water intake (2nd void). See Table 2, for correlation between variables (Spearman test).

Figure 21A, shows that urinary ATP/Cr levels correlate inversely with the mean voided volume determined in a 3-day voiding diary in OAB patients. In other words, urinary ATP/Cr was maximal in OAB patients who reported a mean voided volume less than 100 ml (observed in 9 out of a total of 34 patients). These findings contrast with data from the control group where ATP levels normalized to urine creatinine content (ATP/Cr, pmol/mg) positively correlates with the mean

voided volume (Spearman test, r=0.522, *P*<0.01) (see Figure 21A) and fully agree with the concept that physiological distension of the urothelium releases ATP into the lumen of the bladder (Ferguson et al., 1997; Wang et al., 2005). No other parameter analyzed in this study changed significantly (*P*>0.05) between the three subgroups identified using the mean voided volume as criteria, namely age and urine measurements of creatinine, pH and LDH activity. Thus, finding a correlation between ATP/Cr levels and the mean voiding volume may be taken as a marker for the severity of detrusor overactivity and should be considered of clinical relevance in the follow-up of OAB syndrome. Using 200 ml as a cutoff value, we found that urinary NGF/Cr levels in OAB patients were significantly (*P*<0.05) higher than those found in the control group, but this difference disappeared in less severe forms of bladder overactivity (Figure 21B).

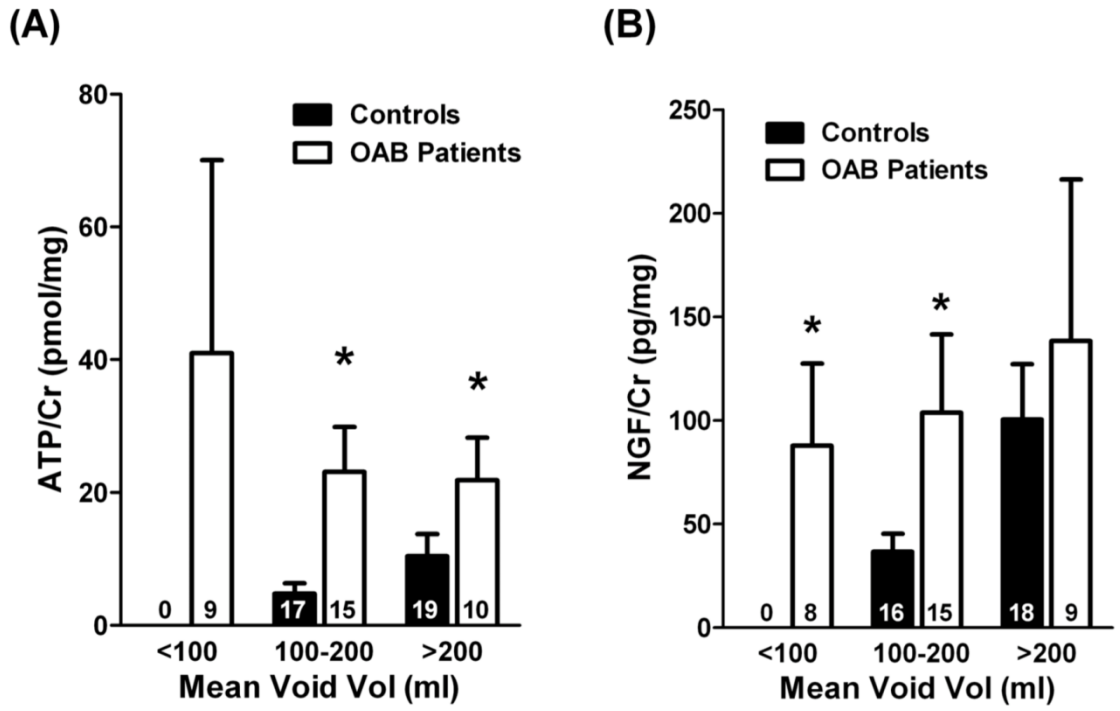

Figure 21. Relationship between urinary (A) ATP/Cr (pmol/mg) and (B) NGF/Cr (pg/mg) content and the mean voided volume determined in a 3-day voiding diary for women with OAB and age-matched controls. Columns are mean values ±standard deviation (SD) from n number of individuals (shown in parenthesis). *P,0.05 (unpaired Student's t-test with Welch's correction) represent significant differences from controls.

In this series, 25% of the women with detrusor overactivity complained of

urgency without incontinence (OAB dry), whereas the remaining group (75%) had urgency urinary incontinence (OAB wet). Figure 22 shows that urinary ATP/Cr, but not NGF/Cr, levels were significantly (P<0.05) higher than controls in women with OAB, despite they have been characterized as OAB dry or OAB wet. No significant differences (P>0.05) were, however, detected in urinary ATP/Cr and NGF/Cr levels among OAB patients of the two subcategories.

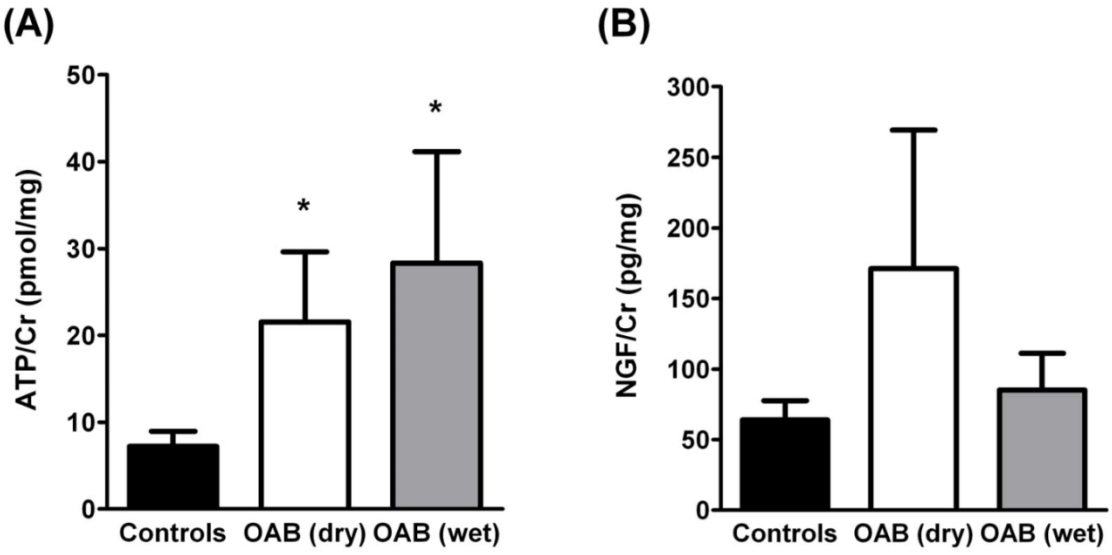

Figure 22. Urinary (A) ATP/Cr (pmol/mg) and (B) NGF/Cr (pg/mg) content in women with detrusor overactivity complaining only of urgency (OAB dry, n = 7) and of urgency associated with urge urinary incontinence (OAB wet, n = 21) as compared to age-matched controls (n=36). Columns are mean values 6 standard deviation (SD). *,**P,0.05 (one-way ANOVA with Dunn's multiple comparison test) represent significant differences from controls and between OAB dry and OAB wet subgroups, respectively.

DISCUSSION

To our knowledge, this is the first study so far designed to evaluate synchronous changes of both ATP and NGF levels in urine samples collected, non-invasively, during voiding at normal desire by OAB women with detrusor overactivity and age-matched controls. Data shows that ATP levels were significantly higher in OAB women with detrusor overactivity than in the control group and this was observed when ATP levels were normalized either to urine creatinine content (ATP/Cr, pmol/mg) or to the urinary volume (ATP.Vol, pmol). A high area under the ROC curve (0.741; 95% CI 0.62-0.86; P<0.001) indicates that urinary ATP/Cr may be a highly sensitive biomarker for discriminating OAB women with detrusor overactivity. In this series, we failed to obtain a similar pattern regarding urinary NGF measurements. Although we found a tendency for

NGF normalized to urine creatinine content (NGF/Cr) to increase in OAB patients as compared to controls, this tendency did not reached statistical significance (P>0.05). Moreover, no significant correlation between urinary ATP/Cr and NGF/Cr levels was found in the samples collected from OAB women with detrusor overactivity.

Interestingly, ATP levels measured in paired samples collected after water intake (2nd void) consistently exceeded those verified in the samples collected upon arrival to the consultation (1st void), both in controls and OAB patients. That is, for a given patient or control, ATP/Cr content in the 2nd void boosted and significantly correlated with the amount detected in the previous (1st) void. This situation was clearly different if one considers NGF/Cr measurements. In keeping with the assumption that urinary NGF content is sensitive to urinary volume variations, we found a wide dispersion between measurements of NGF in paired samples obtained from 1st and 2nd voids, both in controls and in patients with OAB. Our findings fully agree with *in vitro* experiments demonstrating that mechanical distension of bladder urothelium from patients with OAB release significantly more ATP than controls (Kumar et al., 2010). Thus, water intake may increase the sensitivity of urinary ATP measurement as a biomarker of detrusor overactivity in OAB patients (ROC curve area = 0.769; 95% CI = 0.64-0.90; P<0.001). Given that the mean voided volume during the provocative 2nd void significantly increases with respect to the 1st void one would expect that the concentration of the nucleotide should dilute accordingly, yet this was not the case probably because raw ATP production by bladder distension exceeds the water dilution factor, particularly in OAB patients. This was verified even though urinary ATP measured in this study might have been underestimated due to the hydrolysis of the nucleotide by ecto-NTPDases bound to urothelial cells during urine bladder storage. Thus, the more frequent the voids (e.g. OAB patients with detrusor overactivity) the higher urinary ATP levels should be measured. Despite this, we measured higher than control levels of ATP in urine samples collected from OAB patients upon arrival to the consultation (1st void) where no significant differences were detected in the voided volume and inferred time to void (see Table 4). ATP may, thus, be regarded as a novel and important dynamic urinary biomarker of detrusor overactivity which levels reflect adequately bladder distension under pathological conditions.

Several mechanisms may account for increased ATP levels in the urine of OAB women with detrusor overactivity. Hypertrophy and hyperplasia of the urothelium and underlying smooth muscle layers are frequently observed in overactive bladders. Thus, OAB patients might have increased urinary ATP levels produced by thickening of the epithelium taking into account that uro-epithelium is in close contact with the urine and is a major source of the nucleotide in the bladder (Kumar et al., 2010; Silva-Ramos et al., 2010). Whether urothelial cells *per se* release more ATP in OAB patients than in healthy controls remains to be determined. In addition, it is known that parasympathetic nerves from overactive or obstructed human bladder strips release more ATP than controls (Kumar et al., 2010; Silva-Ramos et al., 2010). Enhanced urinary ATP content may also result from impairment of ecto-NTPDase activity observed in cells from OAB patients, as this finding may determine slower nucleotide inactivation kinetics in the bladder wall and its urinary accumulation (Harvey et al., 2002; Silva et al., 2011).

Data from this study demonstrate that urinary ATP/Cr levels correlated inversely with the mean voided volume obtained in a 3-day voiding diary applied to OAB patients. Although the urinary content of the nucleotide corrected to the creatinine level (ATP/Cr) was consistently higher in samples from OAB patients than in age-matched controls, differences tend to decrease when the clinical condition of the patients was less severe (*e.g.* mean voided volume higher than 200 ml). This tendency fully agrees with the lack of differences in the concentration of ATP released during cystometric bladder filling in women with mild bladder pain syndrome and controls (Cheng et al., 2013). Previous reports also demonstrated an inverse correlation between first desire to void and ATP concentration in the voided urodynamic fluid collected from OAB patients during cystometry investigation (Cheng et al., 2010). Both findings are consistent with the hypothesis that ATP has a prominent role modulating the early filling sensation in patients with OAB, which yields to low bladder volumes at first desire to void and symptoms of urgency, frequency and reduced bladder capacity presented by patients of this series. Thus, higher concentrations of ATP would produce early voiding reflexes in OAB patients as suggested in this study. Recently, Sugaya and collaborators investigated the urinary ATP/Cr ratio in a population of 30 women with OAB (aged 69±8 years old) and 6 younger female

controls (aged 37±6 years old) (Sugaya et al., 2009). The ATP/Cr ratio in the Sugaya's study was about one log unit lower than that obtained in our work, probably because cryopreservation of urine samples was not undertaken before readings. These authors did not found statistically significant differences in the urinary ATP content between the two groups, yet patients with higher ATP/Cr ratio had more severe symptoms and worse quality of life scores than the group exhibiting lower urinary ATP content, which fully agrees with our findings. Moreover, they reported that ATP/Cr levels significantly decreased after treatment with anti-muscarinic drugs and the improvement of lower urinary tract symptoms was greater for patients with a high baseline urinary ATP/Cr level (Sugaya et al., 2009). These findings suggest that urinary ATP may be a marker of disease severity and might also be used to predict response to clinical intervention. Preliminary results from our group indicate that symptoms relief after intravesical botulinum toxin type A application in six female patients with detrusor overactivity of this series was accompanied by marked improvement of lower urinary tract symptoms along with a complete regression of urinary ATP/Cr to control levels (unpublished observations).

By far the most studied biomarker of OAB has been NGF, as this neurotrophin is highly expressed in the urothelium and suburothelial region of patients with bladder dysfunction (reviewed in Antunes-Lopes et al., 2011; Kuo et al., 2010; Ochodnicky et al., 2011). There is some evidence from these pilot clinical studies using small patient populations that urinary NGF excretion is also elevated in patients with OAB. This tendency was also observed in this series, yet statistical significance was only obtained for women with the most severe forms of bladder overactivity reporting mean urinary volumes lower than 200 ml in the 3-day voiding diary. However, the peptide may be a downstream element of several bladder pathologies that may occur without urinary urgency (*e.g.* bladder inflammation, outlet obstruction, urolithiasis, neoplasia) (reviewed in Kuo et al., 2010; Ochodnicky et al., 2011). This may cause urinary NGF concentration to vary considerably amongst patients with similar complaints, yet it may still find some utility to assess OAB management as urinary NGF decreased after non-pharmacological and pharmacological treatments (reviewed in Antunes-Lopes et al., 2011; Kuo et al., 2010). Preliminary results published in the literature showed a superior accuracy of brain derived neurotrophic factor (BDNF) over NGF in

diagnosing detrusor overactivity (Antunes-Lopes et al., 2011). In contrast to NGF, a direct release of BDNF from urothelial or smooth muscle cells was never demonstrated and one cannot ignore that other organs besides the bladder may also release neurotrophic factors (Castren & Rantamaki, 2010; Yu et al., 2012). Before adopting neurotrophins as urinary biomarkers of detrusor overactivity, one must also consider several limitations, in addition to the general lack of information about the levels of urinary neurotrophins in normal individuals. Methodological limitations include (a) the variation of urinary NGF and BDNF measurements by ELISA, as this method is not routinely available and requires technical expertise; (b) the binding of neurotrophins to proteins or cells potentially present in the urine of patients; and (c) the specificity of various antibodies against NGF and BDNF precursors, which may add variability to measured urinary neurotrophins. In addition, the reported dependence of urinary NGF content on variations of the voided volume (this study) and bladder distension (Kuo et al., 2010), as well as the lack of studies indicating that there is a clear-cut clinical correlation between neurotrophin levels in urine samples and the severity of detrusor overactivity, may contribute to question their sensitivity to identify individuals with specific bladder pathologies or patients with detrusor overactivity.

In this regard, ATP has a very simple, inexpensive and highly reliable measurement method, the luciferin-luciferase bioluminescence assay. Technical limitation relies on the need for snap-freeze urine samples immediately after collection to avoid ATP degradation during storage. Moreover, urinary ATP is influenced by bacterial infection, kidney dysfunction and malignancy, so care must be taken to exclude such factors. In contrast to neurotrophins, differences between OAB patients and controls are independent on the urinary volume and, in fact, sensitivity of urinary ATP measurements in OAB patients may be enhanced by urine production and consequent bladder filling by water intake. This study proved that there is a positive correlation between urinary ATP levels and clinical symptoms evaluated by simply adding a 3-day voiding diary to analytical tests. Surprisingly or not, we found no statistical significant (P>0.05) differences between urinary ATP/Cr levels in OAB patients complaining of urgency without incontinence (OAB dry) and those reporting incontinence urinary urgency (OAB wet). This supports the notion that incontinence is inconsistently correlated and may have a pathophysiological mechanism distinct from detrusor

overactivity, which might be independent from ATP bladder production. Because several mechanisms may be involved in the pathophysiology of the OAB syndrome, one cannot exclude the benefit of using a panel of urinary biomarkers (including neurotrophins) to target detrusor overactivity and intervention follow-up. Unfortunately, we found no correlation between ATP/Cr and NGF/Cr levels in urine samples collected from woman with OAB in this series.

In conclusion, our results support an important putative role of ATP in the pathogenesis of detrusor overactivity. Data showed that OAB women with detrusor overactivity have high urinary levels of ATP compared to controls and that the nucleotide levels increase with water intake. The increased concentration of ATP in the urine of OAB patients is probably due to enhanced ATP release from proliferating urothelium, since uroepithelial cells are considered the primary source of ATP in the human bladder (Kumar et al., 2004). This hypothesis agrees with previous studies using experimental models of bladder disorders exhibiting enhanced release of ATP from the urothelium (Munoz et al., 2010; Sadananda et al., 2009). Moreover, *in vitro* studies demonstrated that uroepithelial cells from overactive human bladder samples release more ATP than controls and that the release of the nucleotide was resistant to blockade of neuronal activity with tetrodotoxin (Kumar et al., 2010; Silva-Ramos et al., 2010). Decreased ATP metabolism could also explain high levels of ATP in the urine of OAB patients as compared to controls. Reports from our and others groups, have shown that ATP catabolism is hindered in the bladder of patients with outlet obstruction and detrusor overactivity (Harvey et al., 2002; Silva-Ramos et al., 2010), which could also be the case of women with OAB. Thus, our findings are consistent with urinary ATP being a putative dynamic biomarker of detrusor overactivity in women with OAB. Non-invasive urinary ATP measurements may represent an improvement from other diagnostic and follow-up approaches requiring urodynamic investigations. Future studies are needed to further examine whether urinary ATP can also be used as a prognostic factor and to assess the therapeutic outcome in OAB women with detrusor overactivity.

ACKNOWLEDGEMENTS

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ARTICLE 5

INTRAVESICAL BOTULINUM NEUROTOXIN-A INJECTIONS DECREASES URINARY ATP CONCENTRATION IN PATIENTS WITH OVERACTIVE BLADDER SYNDROME

Miguel Silva-Ramos[1,2,3], Isabel Silva[1,2], Daniel Reis[3], José Carlos Oliveira[4], Paulo Correia-de-Sá[1,2]

[1]Laboratório de Farmacologia e Neurobiologia, [2]Center for Drug Discovery and Innovative Medicines (MedInUP), Instituto de Ciências Biomédicas Abel Salazar (ICBAS) - Universidade do Porto (UP), Porto, and [3]Serviço de Urologia and [4]Serviço de Química Clínica - Centro Hospitalar do Porto (CHP), Porto, Portugal.

Author contribution:
Miguel Silva-Ramos: conception and design of the study, patients selection and recruitment, analysis and interpretation of data, statistical analysis, drafting of the manuscript.
Isabel Silva: samples processing/preservation, ATP and LDH determinations, analysis and interpretation of data, statistical analysis
Daniel Reis: acquisition, analysis and interpretation of data.
José Carlos Oliveira: biochemistry and urine analysis.
Paulo Correia-de-Sá: conception and design of the study, project supervision, analysis and interpretation of experimental data, statistical analysis, revision of the manuscript.

ABSTRACT

Objectives. Botulinum neurotoxin-A (BoNT-A) injections affect bladder activity through actions at both sensory and motor pathways of the micturition cycle. BoNT-A effectively inhibit the release of excitatory signaling messengers from non-neuronal cells, namely the urothelium, and from bladder nerve efferents. ATP released into the bladder lumen is significantly decreased by BoNT-A in cultured urothelial cells and in animal models of bladder diseases. Recently, our group showed that urinary ATP significantly increases in women with overactive bladder (OAB) syndrome compared to healthy controls. This prompted us to evaluate whether decreases in the urinary ATP content correlated with the improvement of OAB symptoms in patients injected with BoNT-A.

Methods. Nineteen patients (13 women and 6 men) with OAB refractory to antimuscarinic medication completed the Portuguese version of the overactive bladder questionnaire (OABq) and were asked to void at normal desire into a sterile graduated cup. This procedure was done immediately before and 4 to 8 weeks after injection of 100 U of onabotulinic toxin-A into the bladder wall. Urine samples were analysed to measure the concentration of ATP and creatinine and the activity of lactate dehydrogenase. Infected urine samples were discarded.

Results. Intravesical application of BoNT-A significant reduced OAB symptoms and increased the quality of life (QOL) domain score evaluated in the OABq questionnaire. All patients, but one (5.2%), had a positive response to treatment with BoNT-A. On average, the urinary ATP concentration decreased from 4.82±2.68 nM before to 2.68±2.32 nM (p=0.011) after treatment with BoNT-A. This finding was inversely correlated with the storage capacity of the bladder measured as increases in the voided volumes, which were of 162.6±86.3 ml before and 273.4±144.5 ml after injecting BoNT-A. Urinary ATP concentration showed no correlation with symptoms severity (r=0.219; p=0.368), however higher urinary ATP levels measured before treatment predicted poor improvements in patients' QOL score (r=-0.596; p=0.007) after treatment.

Conclusions. Data suggests that urinary ATP is a dynamic biomarker of bladder overactivity, which may be used to monitor non-invasively, yet accurately, treatment responsivness to BoNT-A injections in OAB patients. The results also show that urinary ATP may have an outcome predictive value, since patients with

higher urinary levels of the nucleotide are more likely to be poor responders to BoNT-A treatment in what concerns the QOL score of the OABq.

INTRODUCTION

Nowadays, BoNT-A injections into the bladder wall are commonly used for the treatment of overactive bladder syndromes refractory to antimuscarinics. Increasing number of studies show that BoNT-A injections enhance bladder capacity, increase the threshold volume of first voiding contraction, and reduce detrusor tone, resulting in significant decrease in episodes of frequency and urgency, thereby significantly improving the quality-of-life (Denys et al., 2012; Nitti et al., 2013; Reitz et al., 2004).

In neurons, BoNT-A binds with high-affinity to synaptic vesicle protein 2 (SV2). Following internalization of this complex, BoNT-A cleaves synaptosome-associated protein of 25 kDa (SNAP-25), which is one of soluble N-ethylmaleimide–sensitive factor attachment receptor (SNARE) proteins mediating fusion of synaptic vesicles to the plasma membranes, resulting in blockage of neurotransmitters exocytosis (Montecucco & Molgo, 2005; Simpson, 1979). The therapeutic outcome of BoNT-A may also be due to its effects on afferent nerves (Apostolidis et al., 2005; Collins et al., 2013). Targeting of BoNT-A to the urothelium has also been demonstrated (Cruz, 2014; Hanna-Mitchell et al., 2015; Khera et al., 2004), although the underlying mechanism of action of BoNT-A in uroepithelial cells is still a matter of speculation even considering that non-neuronal cells can release vesicular cargo through exocytosis.

The urothelium is recognized as an important player on chemical- and mechano-sensing transduction pathways of the urinary bladder, particularly in cases of OAB syndromes. Urothelial cells release ATP in response to both chemical and mechanical stimulations (e.g. bladder distension, increased transmural pressure), which undertakes a series of complex autocrine and/or paracrine amplification responses culminating in the activation of P2X3 receptors in suburothelial afferent nerves that convey sensory information to the central nervous system. It has been demonstrated that urothelial cells from patients with OAB release higher amounts of ATP than controls, which may contribute to urgency and frequency bladder symptoms reported by these patients (Kumar et al., 2010). Interestingly, BoNT-A is known to inhibit the vesicular release of acetylcholine and ATP from efferent nerves, but it can also inhibit ATP release from urothelial cells in animal models of neurogenic and inflammatory bladder diseases (Khera et al., 2004; Smith et al., 2005).

The mechanisms underlying the release of ATP from urothelial cells seem to be complex and are not yet fully elucidated. These may differ depending on the stimulus and whether luminal versus abluminal sides of the urothelium is being evaluated. Release of ATP via pannexin hemichannels has been identified at the apical umbrella cells layer of the urothelium (Beckel et al., 2015; Timóteo et al., 2014). Considerable number of studies also support the vesicular ATP release from both sides of the uroepithelium; for instance, the release of ATP was reduced by incubation with inhibitors of vesicular trafficking and fusion, such as brefeldin A (Sui et al., 2014; Wang et al., 2005) and onabotulinum toxin A (Smith et al., 2005). The involvement of SNARE-dependent mechanisms has also been implicated in the release of ATP from the urothelium (Cruz, 2014).

In this context, urodynamic studies using the isolated rat bladder demonstrated that instillation with BoNT-A decreases ATP release (Collins et al., 2013). Application of liposome-encapsulated BoNT-A into bladder lumen can effectively reduce frequency and urgency episodes in OAB patients (Kuo et al., 2010), Apparently, these effects of BoNT-A are due to its actions on the urothelium almost exclusively. It is, therefore, plausible that the therapeutic effects of BoNT-A injections into the bladder are due not only to their effects on the release of neurotransmitters from bladder nerves, but also via the release of excitatory signaling messengers from urothelial cells. Consequently, BoNT-A injections may decrease ATP release from nerves, both in the detrusor and in the suburothelium, as well as from urothelial cells, which might have impact on the urinary ATP concentration.

We have previously shown that women with OAB symptoms due to detrusor overactivity have higher urinary ATP concentrations compared to healthy controls (Silva-Ramos et al., 2013b). We found a direct correlation between urinary ATP levels and the severity of OAB symptoms. Therefore, we questioned if intravesical BoNT-A injections to treat OAB syndromes resistant to antimuscarinics could effectively reduce urinary ATP concentrations in parallel to improvement of lower urinary tract symptoms and whether changes in the concentration of the nuceotide can be used as a biomarker to predict the therapeutic outcome.

PATIENTS AND METHODS

Patients and procedures.

This study and all its procedures were approved by the Ethics Committees of Centro Hospitalar do Porto (CHP) and of Instituto de Ciências Biomédicas de Abel Salazar of the University of Porto (ICBAS-UP). A total of 25 patients with OAB symptoms and urodynamically confirmed detrusor overactivity, who had in common failure to antimuscarinic therapy, were enrolled between November 2011 and November 2015. Exclusion criteria *ab initium* consist of treatment with BoNT-A in the last 12 months, although during that period of time patients could have been taken anticholinergics and/or the β_3-adrenoceptor agonist, mirabegron. Patients with history of malignancy, pelvic radiotherapy, painful bladder syndrome, bladder outlet obstruction, current urinary tract infections, chronic kidney disease stage > 2, lower urinary tract surgery or any instrumentation in the last 4 weeks, with indwelling catheter or intermittent catheterization, or with post-void residue >100cc, were excluded.

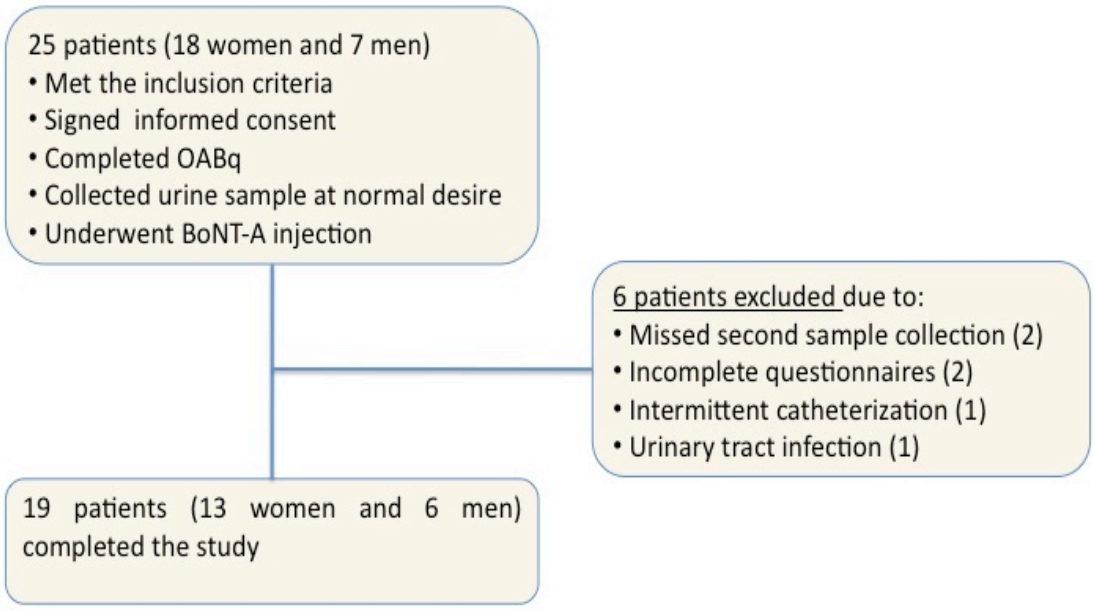


Figure 23. Flow diagram of patients selection.

After informed consent, patients completed OABq (overactive bladder questionnaire) and were asked to void at normal desire to a sterile measuring cup, before and 4 to 8 weeks after BoNT-A injection. Injections were performed under sedation with a flexible or rigid cystoscope. A total of 100 U of BoNT-A (Botox®, Allergan, Irvine, CA, USA) prepared in 10 ml of saline were administered

in 20 sites (0.5 ml each) distributed evenly throughout the detrusor sparing the trigone. Four to eight weeks after the procedure, patients were questioned about adverse events, asked to complete the OABq questionnaire and to void at a normal desire to a measuring cup after which an evaluation of post-void residual volume was estimated by ultrasonography.

Urine samples were tested with a rapid reagent dipstick (Combur-10®, Roche Diagnostics GmbH, Mannheim, Germany) and if negative for nitrites and leucocytes, they were divided into three tubes; one for urine culture, another for creatinine measurement and a third one was immediately snap frozen in liquid nitrogen and preserved at -80ºC until ATP determination. Patients with positive urine cultures were excluded. Nineteen patients (13 women and 6 men) aged 52.8±16.8 years-old completed the study protocol (Figure 23). Patients with less than 25% reduction in bothersome symptomsscore were considered non-responders.

Measurement of urinary creatinine.

The quantitative determination of urinary creatinine was performed in vitro on a Cobas Integra 800 analyser using the kinetic colorimetric Creatinine Jaffe´ Gen.2 assay according to the manufacturers' instructions (Roche Diagnostics GmbH, Mann- heim, Germany).

Measurement of Urinary ATP

Undiluted urine samples were defrosted till 25ºC and afterwards centrifuged at 3000 g at room temperature for 20 seconds to remove cellular debris. The supernatant was separated. A mixture of luciferine-luciferase was added according to the manufacturer instructions using the ATP Bioluminescence Assay Kit HS II (Roche Applied Science Indianapolis, Indiana, USA). ATP detection was evaluated using a multi-mode microplate reader (Synergy HT, BioTek Instruments Inc., Vermont, USA) controlled via BioTek's Gen5^{TM} Data Analysis Software. Sample bioluminescence was compared to that of standard amounts of ATP used in the same concentration range; standard ATP samples were prepared daily. All samples were run in duplicate. The minimum ATP detection limit in these experimental conditions was 10^{-12}M (10^{-16}moles in

100 ml samples) and luminescence correlated linearly to ATP concentration till 10^{-6}M. Each urine sample remaining was used to quantify the lactate dehydrogenase (LDH, EC 1.1.1.27) activity (Keiding, 1974), which is an intracellular enzyme that is commonly used as an indicator of cell integrity.

Statistical Analysis.

Statistical analyses were performed using IBM SPSS Statistics software version 21 (New York, USA). Results are reported as mean values ± standard deviation unless stated otherwise. The paired Student's t-test was used for comparisons between groups after Kolmogorov-Smirnov test showed normality of data distribution. Correlation between variables was analyzed using Pearson test. The correlation between co-variates was performed by multiple regression analysis. $P<0.05$ (two-tailed) values were considered statistically significant.

RESULTS

Treatment of OAB patients with intravesical applications of BoNT-A caused a significant reduction in the bothersome symptoms score and a significant increase in all quality of life (QOL) domains of the OABq questionnaire (Figures 24 and 25). One patient (5.2%) was considered a non-responder, since she reported an increase in the score severity.

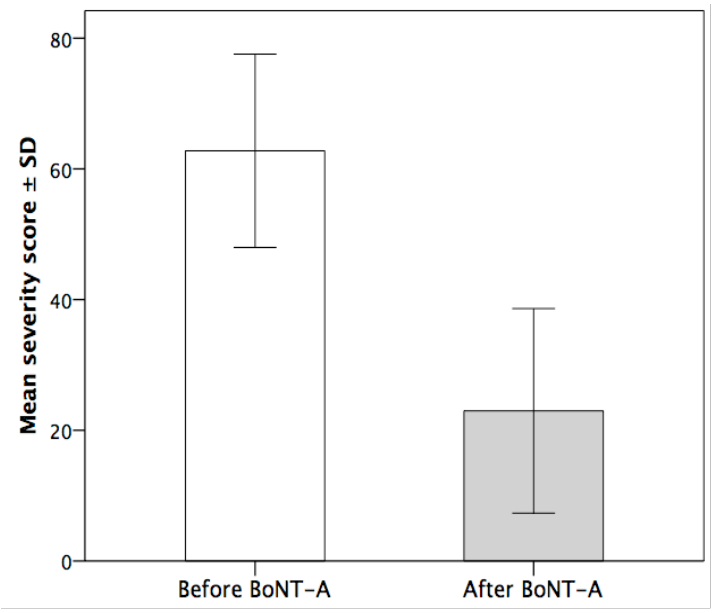


Figure 24. Changes in OABq severity score after intravesical application of BoNT-A. Higher scores mean higher severity (maximum – 100 points). *P<0.05 represents significant differences comparing to the score determined before BoNT-A.

On average, the urinary ATP concentration decreased from 4.82±2.68 nM before to 2.68±2.32 nM (p=0.011) after treatment with BoNT-A (Table 6). There was also an increase in the voided volumes in patients injected with BoNT-A (273.4±144.5 ml) compared to the situation immediately before application of the toxin (162.6±86.3 ml). Notwithstanding this, reductions of urinary ATP concentrations after BoNT-A might not be due to urine dilution.

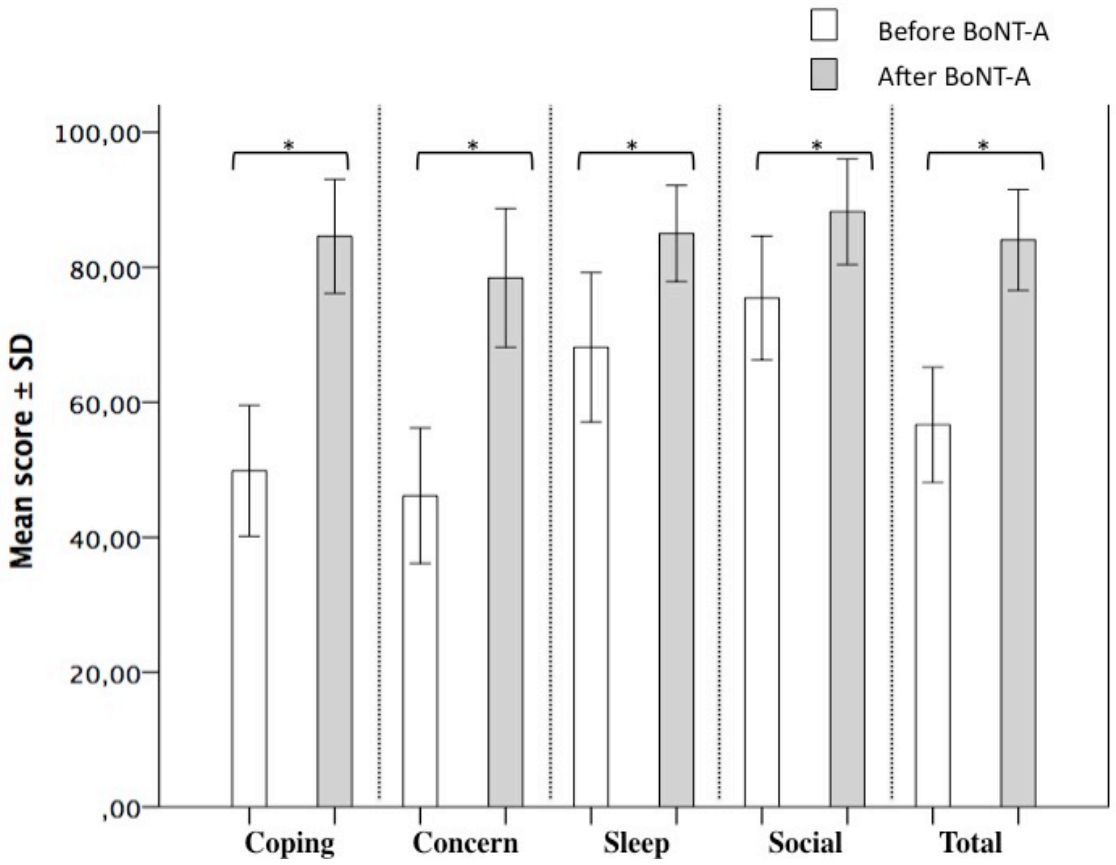


Figure 25. Changes in the QOL domain scores of the OABq before and after intravesical application of BoNT-A. Higher scores mean better QOL (maximum – 100 points). *P<0.05 represents significant differences comparing to the score determined before BoNT-A

As a matter of fact, we showed in a previous study that bladder distension was accompanied by higher urinary ATP amounts (Silva-Ramos et al., 2013b). In agreement with this, Figure 26 shows that albeit there is an increase in the voided volume following BoNT-A treatment we observed a reduction in the absolute ATP amounts (nmol) per volume voided. That is, the slope of the linear

relationship between ATP (nmol) and the volume (ml) of the correspondent voided sample decreased in patients injected with BoNT-A.

Even though we consider that these results are preliminary and the number of patients of both genders are insufficient to take more positive conclusions, it seems that there are differences worth to be reported. Although urinary ATP concentrations of OAB patients before treatment with BoNT-A were similar in both genders (5.1±2.4 nM in females and 4.2±2.4 nM in males; $p>0.05$), the BoNT-A-induced decrease of urinary ATP levels was more notorious in men (Figure 27A). Conversely, the increase in bladder storage capacity apreciated by increments in the voided volume was more evident in women (Figure 27B). Concurrent changes in these two variables, which are characteristic of OAB syndromes, end up in the already mentioned reduction of urinary ATP per voided volume occurring in both genders.

Table 6. Measurements before and after BoNT-A injections.

	Before BoNT-A (mean±SD)	After BoNT-A (mean±SD)	p
ATP (nM)	4.82±2.37	2.68±2.32	**0.011**
Creatinine (mg/dl)	86.9±58.2	76.2±57.9	0.584
Voided volume (ml)	162.6±86.3	273.4±144.5	**0.003**
Post-void residue (ml)	16.0±15.9	48.5±56.4	0.100

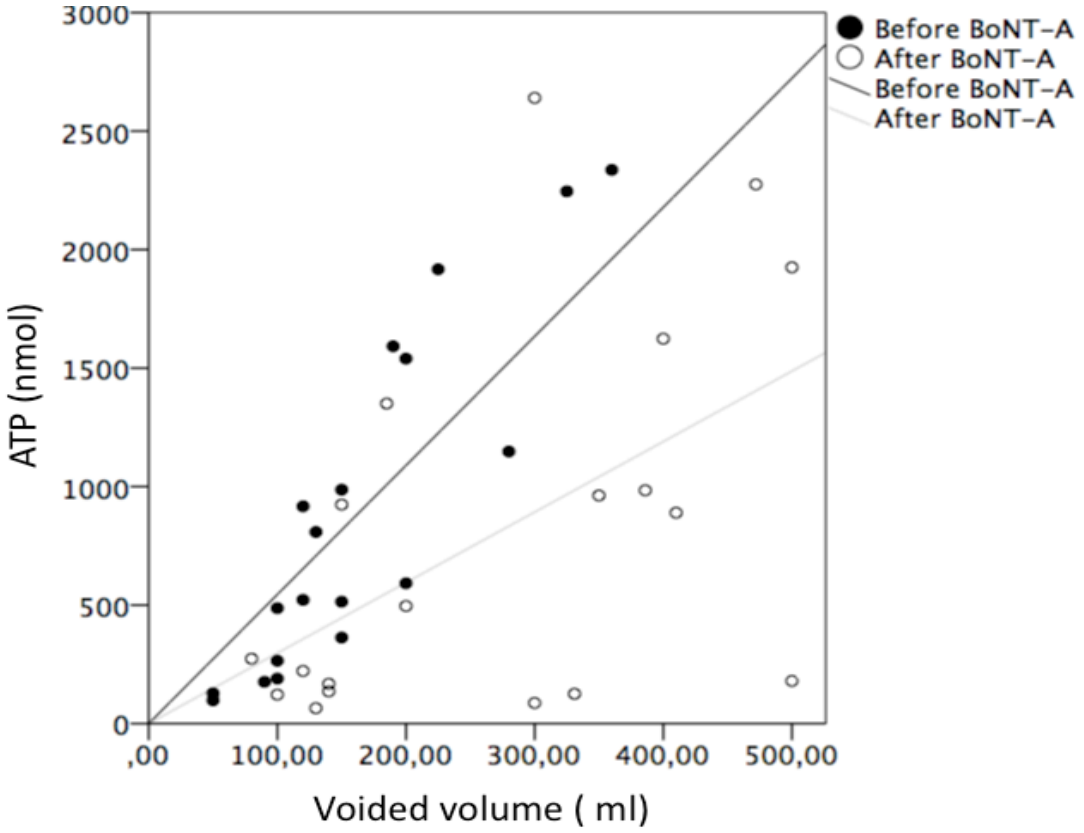
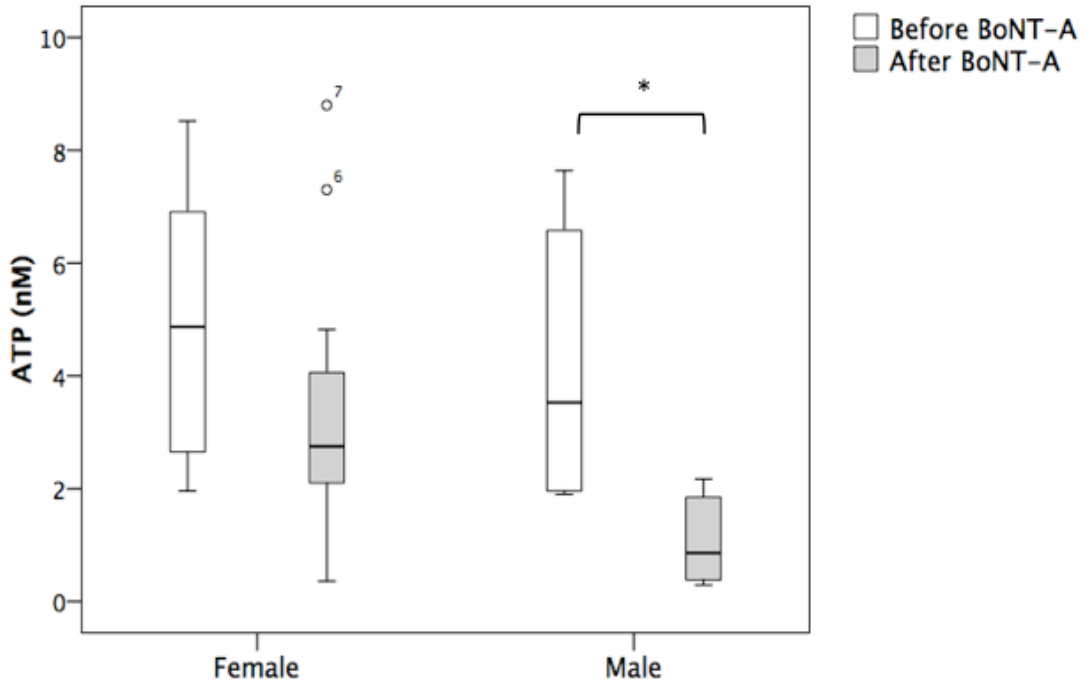


Figure 26. Relationship between urinary ATP amounts (nmol) and voided volumes before and after BoNT-A injections. The slope of the interpolation line between urinary ATP and voided volume is more moderate after treatment with BoNT-A, meaning that for the same voided volume de amount of urinary ATP (nmol) decreased significantly.

Next, we also explored the relationship between urinary ATP (nM) and OAB symptom scores. No significant correlations were detected between symptoms severity and/or QOL scores and urinary ATP concentrations before treatment with BoNT-A. Nevertheless, the urinary ATP concentrations correlated positively with the severity symptoms score (r=0.697; p=0.001) and negatively with the total QOL score (r=-0.644; p=0.003) after treatment. Notwithstanding the fact that urinary ATP concentration showed no correlation with symptoms severity (r=0.219; p=0.368), higher urinary ATP levels measured before treatment with BoNT-A is a predictor of poor outcome concenrning patients' quality-of-life (QOL) (r=-0.596; p=0.007).

A



B

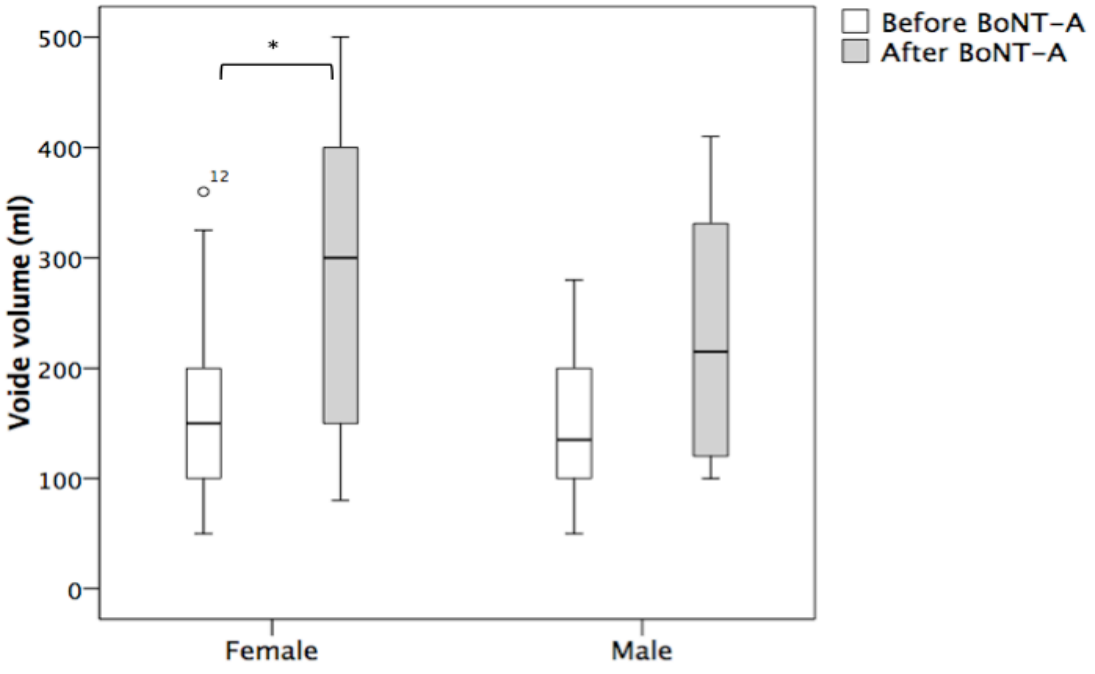


Figure 27. Urinary ATP (nM) (A) and voided volume (B) before and after BoNT-A injections in males and females. Although urinary ATP concentrations diminished after treatment in both genders, only in males the reduction was statistically significant. Conversely, the increase in the voided volume after treatment was more pronounced in females. *P<0.05 represents significant differences comparing to the values determined before BoNT-A.

DISCUSSION

Mounting evidences support a role for ATP in the physiology of the lower urinary tract and this transmitter has strong implications in the pathophysiology of OAB. Although it has been well documented the release of ATP from efferent nerve fibres innervating the bladder and that the nucleotide contributes to detrusor tone, particularly in pathological conditions, more recent studies highlighted its role as mediator of sensory inputs generated by chemical and/or mechanical stimulation of the bladder, like for instance the distension-induced voiding reflex. Upon stretch, ATP is released by the urothelium to both luminal and abluminal sides. Interestingly, the extracellular accumulation of ATP in the bladder lumen may be 50-fold higher than the concentration of the nucleotide in the abluminal side (Wang et al., 2005) and this may be due to an heterogenous distribution and activity of nucleotide release sites and metabolizing ecto-NTPDases, which are responsible for its extracellular catabolism (see e.g. Silva-Ramos et al., 2015b). At urothelial and suburothelial layers, ATP may activate P2X3 receptors (or P2X2/3 heteromers) on sensory afferent nerves, while luminal ATP may have autocrine and/or paracrine actions regulating its own and other mediators release, as well as membrane trafficking within umbrella cells. ATP released into the bladder lumen may be detected in the urine (Sugaya et al., 2007). Therefore, measuring urinary ATP concentrations may give insights on the purinergic signaling tone under certain clinical conditions affecting the lower urinary tract in humans. For instance, our group demonstrated that urinary ATP concentrations are significantly higher in women with OAB syndrome and the levels of the nucleotide positively correlated with symptoms severity (Silva-Ramos et al., 2013b). In this study, we show that urinary ATP decreases in OAB patients after treatment with BoNT-A, supporting the idea that clinical improvements following BoNT-A injections may be mediated, at least partially, by decreases in the release of ATP from the urothelium. As a matter offact, at least in awake, freely moving rats, ATP instillation into the bladder lumen induces detrusor overactivity in a concentration dependent manner (Pandita el al, 2002), therefore inhibition of ATP release or blockade of its receptors may contribute to ameliorate bladder overactivity.

Normalization of urinary biomarkers to urine creatinine is a common procedure. In a previous report, we demonstrated that urinary creatinine did not

correlate with urinary ATP concentrations and, therefore, this normalization was meaningless (Silva-Ramos et al., 2016b). Again, in this series we found no significant modifications on urinary creatinine concentrations in samples collected before and after treatment of patients with BoNT-A. For this reason, results are presented in urinary ATP concentrations without no further correction.

Regarding gender differences found in this series of patients, our preliminary data show that reduction of urinary ATP concentrations following treatment with BoNT-A was clearly more pronounced in males than in females. Previously, other groups reported gender differences in urinary ATP concentrations, favouring higher baseline values in females (Gill et al., 2015; Sugaya et al., 2009). To our knowledge no plausible explanation exists for these observations, yet they might have impact on the magnitude of the response to BoNT-A. We found no significant differences in the concentration of urinary ATP before treatment with BoNT-A among males and females with OAB. However, the urinary ATP concentration before BoNT-A treatment in women in this study was lower than the one published previously by our group (Silva-Ramos et al., 2013b). This difference may be explained by the fact that in the previous study women with OAB were not undergoing any treatment, whilst in this study all women were under pharmacological treatment with antimuscarinics and/or mirabegron. This lower than expected urinary ATP found in women may explain why return of urinary ATP concentration to basal levels following BoNT-A injections was more significant in OAB men than in women, i.e. the gap between baseline and disease levels of ATP in men's urine was higher than that observed in women turning the variation of the concentration of the nucleotide after BoNT-A more evident in men presenting constitutively lower levels of urinary ATP in control conditions than women (see above). Another possible explanation may be due to the greater increase in voided volumes observed in females compared to males after BoNT-A treatment. In women treated with BoNT-A, bladder distension to accommodate more urine may partially counteract the expected decrease in urothelial ATP release caused by the toxin, because on its own mechanical tension and/or bladder stretch are powerful inductors of the nucleotide outflow (Ferguson et al., 1997) leading to its accumulation in the urine (Silva-Ramos et al., 2013b).

Several studies have shown the efficacy, safety, and tolerability of BoNT-A for the management of OAB refractory to antimuscarinics (Patel et al., 2006). In keeping with this, here we verified an overall improvement on the severity symptoms and in the QOL score of OAB patients treated with BoNT-A. Probably because the number of patients recruited for this study was limited, we found no significant correlation between the improvement of the symptoms score and urinary ATP concentrations. The analysis of these parameters may be biased by the inclusion of both males and females in the same group of OAB patients. Notwithstanding the fact that we could not find a clear correlation between urinary ATP concentrations and OAB symptoms severity or the total QOL score just before BoNT-A treatment, this gained significance after application of the toxin. In patients treated with BoNT-A a significant direct correlation between prevailing symptom severity and urinary ATP content was observed. This is in agreement with previous studies from our and other groups showing that more symptomatic OAB patients exhibit higher urinary ATP (Silva-Ramos et al., 2013b; Sugaya et al., 2009). It might happen that highly symptomatic patients refractory to previous medications exhibit initial urinary ATP levels beyond a threshold that hardly correlates with any clinical findings, but this bias may be overcome when the ATP concentration and symptom scores return towards a controlled disease condition.

There are other reports showing a reduction of urinary ATP concentraions in OAB patients treated with anticholinergics (Sugaya et al., 2009), and the decrease of urinary nerve growth factor following BoNT-A injections (Liu et al., 2009). Nevertheless, in any of these publications the urinary biomarker could predict treatment outcome. We attempted to explore this hypothesis in this study. Since only one patient was considered a non-responder, it is unreasonable to compare urinary ATP between responders to non-responders. Therefore, we decided to test correlations between urinary ATP concentrations and the clinical outcome determined by the OAB questionaire. No correlation between urinary ATP and the severity of symptoms score was verified, yet patients with higher urinary ATP concentrations before treatment performed worse in the QOL score after BoNT-A injections. This indicates that urinary ATP concentration can predict treatment outcome with BoNT-A, at least regarding restoration of QOL. From the clinical view point, the possible predictive role of urinary ATP is appealing but needs further investigations to access its extent.

CONCLUSIONS

Data from this study indicate that bladder injections with BoNT-A effectively reduce urinary ATP concentrations in patients with OAB, a situation that was more pronounced in men than in women. It appears that patients with higher urinary ATP levels on admission exhibit poorer outcomes in QOL scores after BoNT-A treatment.

CHAPTER 4
DISCUSSION

“To much agreement kills a chat”

Eldridge Cleaver

4.1 OVERALL DISCUSSION

Last decade assisted to significant changes on the focus of LUTS research towards the bladder, as major organ implicated in the pathophysiology of these syndromes. Purines (ATP and ADO) are paramount molecules in cell signalling in the normal human bladder, yet increasing evidences implicate changes in the purinergic cascade as a major source of LUTS associated with bladder dysfunction. Data presented in this thesis further support this theory and forecast a chief role of purines in the diagnosis, treatment and follow-up of LUTS.

The results shown in Section 3.1 fulfil some gaps in our knowledge concerning the mechanisms underlying the increase in the purinergic tonus commanding detrusor activity in the bladder of BPH patients. We presented direct evidences that ATP is released in higher amounts from detrusor strips of the bladder of BPH patients stimulated electrically. Moreover, we demonstrated that higher extracellular ATP accumulation may result from deficient breakdown of the nucleotide due to impairement of NTPDase1/CD39 activity in the detrusor of obstructed BPH patients. In addition to this, we also found the the activity of ecto-5'-nucleotidase/CD73, the rate limiting enzyme for adenosine generation from released adenine nucleotides, was hindered in the detrusor of obstructed human patients resulting in poor adenosine formation. While ATP accumulation directly increases detrusor muscle tension through the activation of ionotropic P2X1 receptors, the nucleotide indirectly potentiates the release of ACh through an action via P2X2/3 receptors on cholinergic nerve terminals, which activity is upregulated in the bladder of obstructed BPH patients. The activity of prejunctional facilitatory P2X2/3 receptors in obstructed bladders is unrestrained because the compensatory A_1 inhibitory tonus is hindered in these patients due to the lack of adenosine formation. Overall, these features end-up in a situation where both cholinergic neurotransmission and purinergic signalling are hyperactive leading to detrusor overactivity. All these mechanisms are summarized on Figure 28.

Using a neurochemical methodology that allows direct measurements of radiolabeled ACh, we demonstrated here for the first time that urothelium-denuded detrusor strips from BPH patients release higher amounts of ACh than samples from control organ donors with no evidence of LUTS. This finding is very

important, since until now the influence and sensitivity of the human detrusor to cholinergic agents in the context of BOO was still a matter of debate in the literature. In fact, a lot of conflicting results have been published (Michel & Barendrecht, 2008) concerning detrusor function after BOO. In animal models, detrusor contractions induced by electrical field stimulation may reflect the closest situation to an *in vivo* myographic recording, yet discrepancies exist in the literature where the majority of the studies report either a reduced or an unchanged contractile response in BOO (Michel & Barendrecht, 2008). Studies reporting contractions induced by muscarinic agonists and KCl in animal models of BOO also did not help much because their results were also inconsistent. Data concerning human detrusor contractility changes induced by BOO are scarce; the existing results suggest no significant changes on EFS- or carbachol-induced detrusor contractions observed in detriment of a significantly more powerful atropine-resistant purinergic component (Bayliss et al., 1999; Harvey et al., 2002), as we showed in this study. These nuances on cholinergic neurotransmission in obstructed detrusor samples from animal models and humans (see discussion in Section 3.1) probably reflect not only the type of obstruction, but also the time and degree of obstruction, making comparisons very difficult to establish and, sometimes, anecdotal. We reported here that detrusor strips from obstructed BPH patients were less sensitive to exogenous ACh, which may be due (1) to a reduction of detrusor contractility associated with bladder stifness and/or (2) to muscarinic receptors down-regulation resulting from increased amounts of ACh released from cholinergic nerves. Although we have no data measuring KCl-induced detrusor contractions it is plausible that some structural changes, like the smooth muscle hypertrophy and increase in the deposition of extracellular matrix proteins, may contribute to this finding. On the other hand, the linkage between excessive ACh release from stimulating cholinergic nerve efferents and downregulation of muscarinic receptors on detrusor smooth muscle fibres may explain the significant number of patients with BPH that fail to respond to competitive antimuscarinic agents used to control detrusor overactivity. This feature prompts for looking at alternative signalling pathways to tackle with this clinical situation, which according to the data presented in this thesis may be the purinergic cascade. It is our opinion that the re-equilibrium of the cholinergic nerve activity with P2X2/3 receptor antagonists or adenosine A_1 receptor activators, with

or without blocking P2X1-mediated detrusor contractions, could restore the sensitivity of the detrusor to antimuscarinic agents or even control on their own detrusor overactivity in BPH patients.

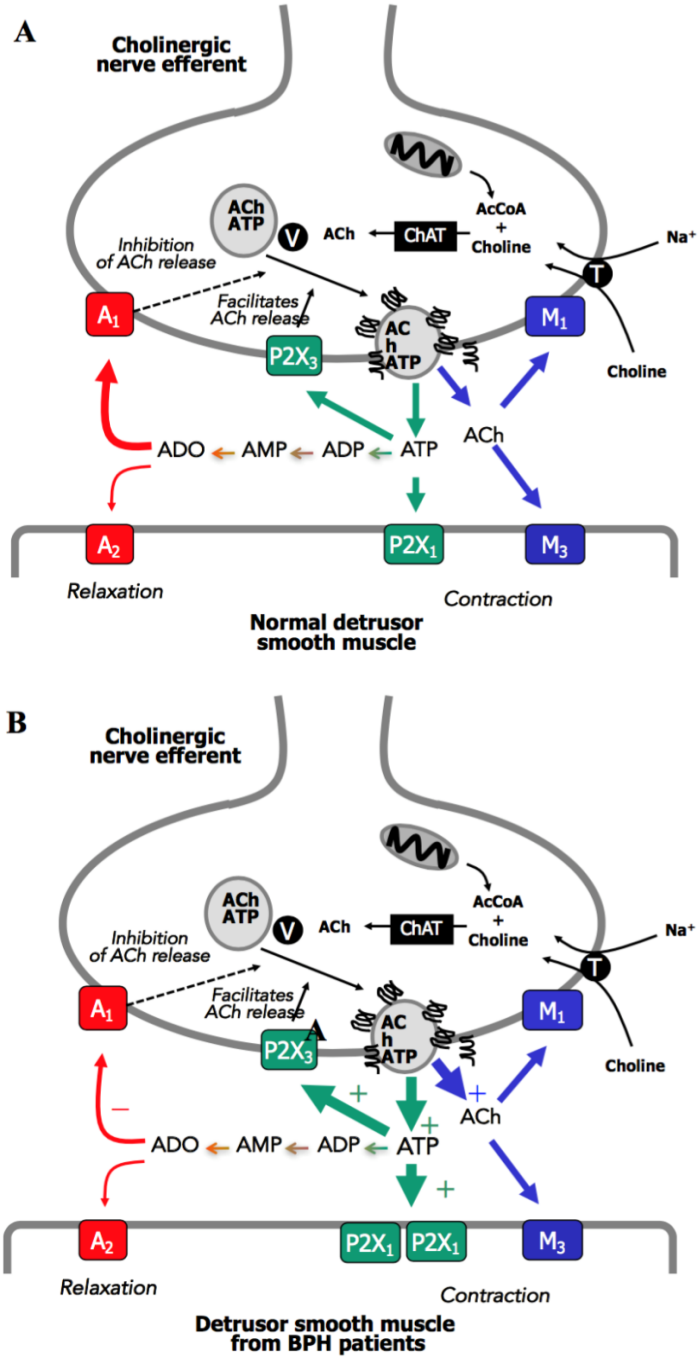


Figure 28. Schematic representation of actions of ATP and ADO on neuromuscular transmission in the human detrusor of healthy individuals (A) and of BPH patients (B). In the detrusor of BPH patients there is an increase in the release of ATP and ACh from activated cholinergic nerves. ATP is slowly inactivated and ADO formation is hampered in these patients resulting in transmitter release facilitation due to activation of presynaptic excitatory P2X2/3 receptors and deficient adenosine A_1-receptors-mediated inhibition. At the postjunctional level, surplus ATP accumulation favors detrusor overactivity through the activation of P2X1 receptors, whose expression is upregulated in BPH patients.

The mechanisms underlying the increase in the purinergic tone in BOO patients are far from being completely understood. The extracellular amount of purines increase dramatically in ischemic conditions and this has been proven in several tissues. Data from experimental animals support the hypothesis that the detrusor of obstructed patients experiments periods of ischemia during voiding, which tend to be more prolonged with disease severity. These ischemic periods cause pH reduction in the detrusor along with oxidative stress and the expression of tissue damaging molecules in the bladder wall, which may lead to an enhancement of the purinergic tone. The purinergic signalling cascade does not depend only on the amount and type of purines released by stressed cells, but also on the expression and activity of extracellular metabolizing enymes, ecto-NTPDases and ecto-5'-nucleotidase, which are very sensitive to environmental alterations, including pH and co-factors like Ca^{2+} and Mg^{2+}. Thus, changes in tissue pH may be enough to explain the deficits in the activity of membrane-bound NTPDase1/CD39 and ecto-5'-nucleotidase/CD73 observed in obstructed BPH patients. As a consequence of these enzymatic changes, ATP tend to accumulate in the detrusor and adenosine formation becomes deficient. In this study, we showed that these deficits could be reverted by applying exogenously apyrase (the homolog of NTPDase1/CD39) or by blocking adenosine cellular uptake and/or deamination with dipyridamole and EHNA, respectively. All of these compounds contributed to normalize purinergic neuromodulation back to control conditions where the excitatory actions of ATP (mediated mainly by ionotropic P2X receptors) are balanced by its retaliatory metabolite, adenosine (via the activation of inhibitory A_1 receptors).

Even though these effects were all tested in urothelium-denuded detrusor strips and, thus, restricted to the detrusor neuromuscular synapse, one may speculate that if ATP reaches high enough concentrations it may also target the P2X2/3 or P2X3 receptorts located in suburothelial sensory nerve afferents and other cell types involved in sensory inputs to the CNS and local reflexes that promote detrusor overactivity. Moreover, the lack of endogenous production of adenosine in obstructed bladders may unbalance ATP excitation at different cellular levels (urothelial cells, suburotelial nerves, myofibroblasts and detrusor smooth muscle fibres) besides the main inhibitory action of the nucleoside on cholinergic nerve efferents. In reaction to endogenous adenosine deficits, we

showed that cholinergic nerves of the bladder of obstructed BPH patients exhibited higher amounts of the inhibitory A_1 receptor. This opens another window of opportunity to control ATP-mediated excitation and attenuate cholinergic neurotransmission in patients with detrusor overactivity due to BPH using selective adenosine A_1 receptor agonists, like the one we have used in this study (R-PIA). Within this context, it is tempting to speculate that drugs targeting the inhibitory A_1 receptor or that bolster endogenous adenosine accumulation (e.g. nucleoside uptake blockers and/or ADA inhibitors) might have potential benefits in the treatment of LUTS, in parallel to the already identified P2X receptor antagonists acting on sensory neurons of the bladder (Gever et al., 2010).

Targeting ATP catabolism as not been clinically explored yet in the context of urological disorders, but seems a promising endeavor. As mentioned before, ATP breakdown is compromized in obstructed bladders of BPH patients and this may contribute significantly to the increase in ATP-mediated effects underlying LUTS in these patients. This feature explains why the enzymatically-stable ATP analogue, α,β-met-ATP, has similar contractile effects on control and obstructed detrusor strips, whereas ATP was more powerful in obstructed samples (Harvey et al., 2002). According to our enzymatic kinetic data and functional reports in the literature (Harvey et al., 2002), NTPDase1/CD39 seems to be the main responsible for the extracellular ATP breakdown in the human detrusor. This was evidenced because ATP was rapidly converted into AMP, without significant accumulation of ADP. Moreover, our results showing that NTPDase1 activity is impaired in detrusor strips of BPH patients are in agreement with data from the Harvey group demonstrating that the ATP breakdown sensitive to ARL67156, a preferential NTPDase1 and NTPDase3 inhibitor without any measurable effect on NTPDase 2 and NTPDase8 (Levesque et al., 2007), was significantly less in detrusor samples from overactive and obstructed bladders than from control individuals (Harvey et al., 2002). Even with this limitation, we were able to significantly increase ATP-mediated contractions of detrusor strips from BPH patients using ARL67156. Thus, we hypothesize that strenghtening of the purinergic tone using NTPDase inhibitors may be therapeutically relevant to overcome detrusor underactivity revealed at later stages of bladder obstruction due to BPH, particularly in elderly men. This may be a suitable alternative to the use of non-suppported cholinomimetic agents, because cholinergic sensitivity of

detrusor smooth muscle fibers may be dramatically impaired in these patients, as we demonstrated in this study (see below).

Interestingly, mucosal ATP is hydrolyzed via a different set of ecto-NTPDases; ATP breakdown is more pronounced in the abluminal than in the luminal side of the urothelium (Figure 29). Using immunofluorescence confocal microscopy, our group showed that NTPDase2 is the most abundant enzyme being expressed in all layers of the urothelium and in the submucosa. In the case of ecto-5´nucleotidase/CD73, this enzyme has a clear assimetric distribution staining more abundantly in the basal layer of the urothelium, suburothelial connective elements and detrusor smooth muscle fibers (Correia-de-Sá, 2010). This distribution pattern is in agreement with HPLC kinetic experiments showing that ATP breakdown yields significant amounts of ADP and adenosine in the abluminal side of the urothelium (Silva-Ramos et al., 2015b). Taking this into consideration, one may hypothesize that the role of adenosine must be more prominent in the abluminal side and quite minimal in the luminal side of the urothelium.

Likewise, Ussing chamber experiments using the bladder mucosa of rabbits showed that upon stretch the amount of ATP was higher in the luminal compartment, whereas adenosine accumulates in higher amounts in the abluminal side of the urothelium (Lewis & Lewis, 2006; Wang et al., 2005) . Although there are reports indicating that adenosine inhibits ATP release in the abluminal side (Dunning-Davies et al., 2013; Yu et al., 2006), less is known about its action on suburothelial structures. Anyway, increasing adenosine and diminishing ATP concentrations at the mucosal level by favoring ATP breakdown may be of therapeutic value in the treatment of LUTS.

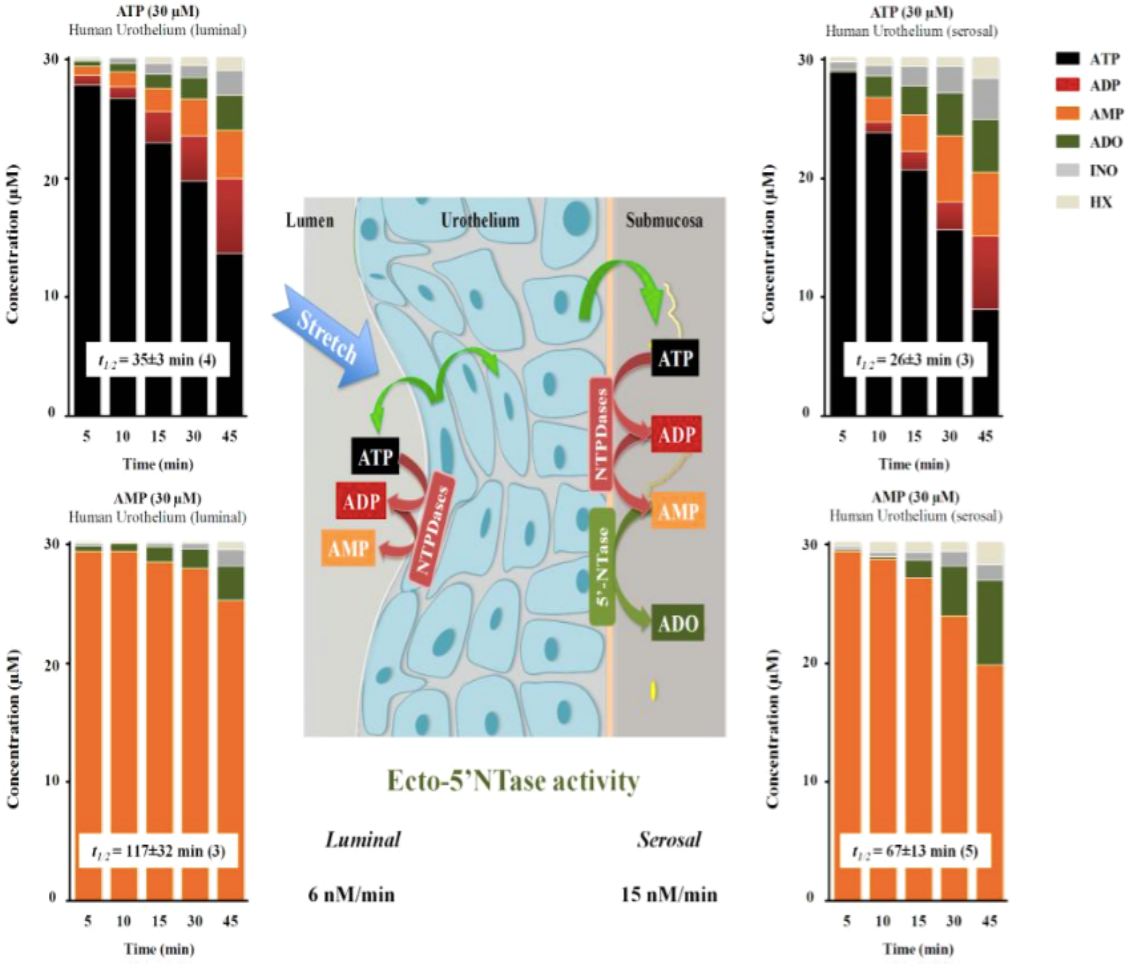


Figure 29. The catabolism of adenine nucleotides and adenosine formation by ecto-5'-nucleotidase is faster in the serosal than in the luminal side of the human urothelium. Illustrated is the time course of the extracellular catabolism of adenine nucleotides and adenosine formation in the isolated urothelium from organ donors (control individuals). ATP and AMP (30 µM) were added at zero time either to the luminal or to the serosal side of the human urothelium. Samples (75 µL) were collected from the incubation fluid at the indicated times on the abscissa. Each collected sample was analyzed by HPLC with UV detection to separate and quantify ATP (black), ADP (red), AMP (orange), ADO (green), INO (dark grey) and HX (light grey). Average results obtained in 3-5 individuals. The calculated half-life time ($t_{½}$) for each initial substrate is shown for comparison (Silva-Ramos et al., 2015b)

It is also worth noting that in the human bladder the ATP catabolism is mimimal at the mucosal side, similarly to what happens in bladder mucosa of rabbits (Wang et al., 2005). Although the role of ATP released on the abluminal side is better documented, luminal ATP also plays a role in the control of micturition. It is, however, not clear if luminal ATP can difuse through the urothelial barrier to activate suburothelial targets. Anyway, it has been shown that ATP instilled into the bladder induces bladder overactivity in the rat (Pandita & Andersson, 2002). Furthermore, intravesical perfusion of apyrase increased the

intercontraction interval in the anesthesized rat, whereas the ecto-ATPase inhibitor ARL67156 decreased it (Beckel et al., 2015; Timóteo et al., 2014). Given the size and hydrophilic nature of these compounds, it is unlikely that they could cross the urothelial barrier to act on sub-urothelial targets. It is more plausible that luminal ATP activates receptors on umbrella cells, since they express several P2X and P2Y receptors, both in rats (Birder et al., 2004; Chopra et al., 2008) and in humans (Silva et al., 2015). In agreement with this, our group showed that instillation of UDP or its analog PSB0474 into the rat bladder increased the voiding frequency through increase of luminal ATP concentrations (Carneiro et al., 2014). Thus, luminal ATP may activate receptors in umbrella cells creating a vicious cycle ending up to increase the release of ATP and other neurotransmitters from the basolateral surface of the urothelium that participate in bladder chemical- and mechano-sensations.

Another consequence of the slow ATP breakdown in the luminal side of the urothelium is the possibility to have direct access of its concentration in voided urine, since significant changes in urinary ATP over time due to enzymatic metabolism are unlikely. Likewise, differences seen *in vitro* in the release of ATP from the mucosa of patients with LUTS and controls are expected to be reflected in the urine, as we show in Section 3.2. In spite of this opportunity, the urinary concentrations of ATP have not been studied systematically. As a matter of fact, the actual source of the ATP found in urine is uncertain. Some of the ATP may come from the kidney, yet most authors agree that the bladder is the major source of urinary ATP. Supporting this idea are the following arguments:

1. Significant amounts of ATP are released upon bladder tissue stimulation. Most of this ATP comes from the urothelium directly into the luminal side (Ferguson et al., 1997; Wang et al., 2005).
2. In vitro bladder distension causes ATP release; results from this thesis show that the urinary concentration of ATP is directly proportionate to the voided volume (i.e. bladder distension). This was evidenced in both women and men.
3. There is no relation between urinary ATP and the concentration of creatinine in the urine.
4. Urinary tract dysfunctions impact on urinary ATP concentrations.

5. Treatment of LUTS with antimuscarinics or alpha-blockers (Sugaya et al., 2009), as well as with with intravesical BoNT-A injections (this thesis), decreases urinary ATP concentrations.

Within the bladder, efferent nerves and uroepithelium are regarded as the most common suppliers of ATP. Mounting evidences point towards the urothelium as the main source of urinary ATP, given that evoked release of ATP is almost abolished in bladder strips without urothelium (Kumar et al., 2004; Munoz et al., 2010). Sun and col. demonstrated for the firt time that ATP could be released by stretching cultured urothelial cells from patients with interstitial cystitis/painfull bladder syndrome and this could be translated into higher concentrations of the nucleotide in the urine (Sun et al., 2001). There are other reports proving that ATP can be released *in vitro* upon stimulating (by EFS or stretching) the urothelium of patients with OAB (Kumar et al., 2010) or BPH (Silva et al., 2015; Sun et al., 2002). Here, we proved that the urine of patients with OAB and BOO collected by voiding at normal desire exhibited higher ATP concentrations than that from healthy controls. This lead us to hypothesize that urinary ATP could be a highly-sensitive non-invasive biomarker of bladder dysfunctions.

In fact, there is an unmet clinical need for non-invasive tests designed to help the diagnosis of detrusor overactivity and BOO in patients with LUTS. Searching for urinary biomakers is becoming a popular endeavour and several of them have been attempted for OAB in addition to ATP. These include NGF, BDNF, prostaglandins, cytokines and glycosaminoglycans (GAG) (Alkis et al., 2015; Bhide et al., 2013). Urinary neurothrophins are perhaps the most studied urinary markers of OAB (Fry et al., 2014). Both NGF and BDNF have been shown to be elevated in patients with OAB, to correlate with disease severity and to be sensitive to treatment response (Antunes-Lopes et al., 2013). Although in our hands urinary ATP was superior to NGF as a diagnostic marker of OAB, ATP and BDNF have ROC AUC´s in the same range (Antunes-Lopes et al., 2011). The problem is that the release of BDNF has never been reported in the human bladder and urinary levels of this neurotrophin may reflect systemic disease conditions. Interestingly, urinary ATP correlates with symptoms severity, it significantly decreases with BoNT-A treatment and may predict treatment outcome, although larger studies are needed to validate this assumption. Looking

at the several urinary biomarkers for OAB published in the literature, it is noticeable that they are related to different patophysiologic processes, such as nerve growth, inflammation, urothelial signalling or a combination several of these mechanisms. So, in accordance to the multifactorial nature of OAB, the development of a panel with several urinary biomarkers could be worthwhile, mainly if they could give insights onto the etiology of OAB in a given patient and/or predict treatment oucomes.

Another important issue we came across during this research was the diference detected in urinary ATP levels between men and women. This has been reported before (Gill et al., 2015; Sugaya et al., 2009), although no valuable explanation has been advanced. One hypotesis would be the higher death cellular content in the urine of females, which is in accordance with higher urinary DHL activity and the presence of large desquamative cells in the sediment. It is noteworthy the fact that in our studies urine samples were centrifuged to remove cellular debris and only aliquots from the supernatant were collected for analysis. Anyway, mean urinary ATP concentrations are significantly higher in women rendering impossible to compare groups with different genders (Figure 31).

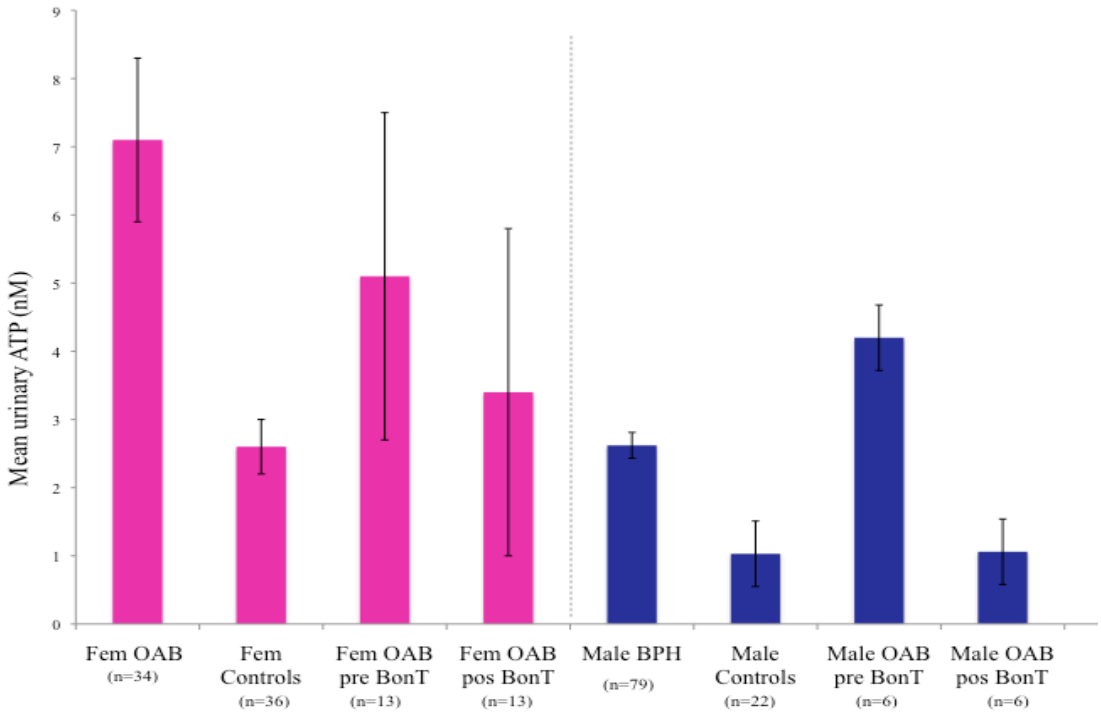


Figure 31. Urinary ATP (nM) distributed by gender. Bars represent mean values ± standard deviation.

Men, on the other hand, exhibit less urinary ATP variability and, for this reason, urinary ATP accuracy as a biomarker may be greater in men. Here, we proposed for the first time that urinary ATP may be a marker of detrusor competence in males with BOO due to BPH. The notion that we can diagnose obstruction by an simple non-invasive urine test is attractive, particularly considering that diagnosing obstruction is a difficult and some times cumbersome process. Since the prevalence of detrusor underactivity is increasing (nowadays, estimated to range between 11-40%) (Jeong et al., 2012; Thomas et al., 2004) and these patients do not benefit from surgical treatment of BPH (Thomas et al., 2004), there is a urge medical need to correctly identify these individuals. Although this issue has not been directly addressed in our studies, assuming that ATP found in the urine is related to bladder wall tension, urinary ATP may be able to distinguish patients with detrusor underactivity among those with BOO showing low urine flow rates and near-control urinary ATP concentrations. Conversely, we presume that patients exhibiting higher than normal urinary ATP have detrusor overactivity. Confirmation of the discriminative power of urinary ATP in LUTS obviously requires integration of this information with standard invasive urodynamic tests (e.g. pressure-flow study).

4.2 CONCLUSION AND FUTURE PERSPECTIVES

Data presented in this thesis highlights the relevance of the bladder purinergic system in the pathophysiology of LUTS in patients with BOO and in patients with OAB. Additionaly, we gathered information to propose that the pharmacological manipulation of the purinergic cascade might be usefull in the treatment of detrusor underactivity, but this hypothesis requires further investigations in the near future.

In patients with BOO due to BPH, the enhanced purinergic tone in the bladder may be interpreted as a critical response enabling the detrusor to generate more pressure to overcome the outflow obstruction. However, on the other hand, excessive purinergic signaling may also be regarded as a pathogenic factor underlying detrusor overactivity, which might be responsible for storage symptoms that have a negative impact in QOL in these patients. In this context, any therapeutic strategy designed to correct the unbalance between ATP and

ADO mediated actions might be beneficial in patients with bladder overactivity due to BOO. These strategies may include (1) the activation of inhibitory A_1 adenosine receptors, either directly with selective agonists or indirectly by increasing the availability of the endogenous nucleoside by inhibiting ADA or the uptake transport system, or (2) blockade of the excitatory P2X2/3 receptors. Taking into consideration that patients with BOO exhibit higher urinary ATP concentrations than healthy individuals, data from this thesis strenghten the role of urinary ATP as a putative noninvasive biomarker of detrusor competence in patients with BOO.

The enhanced purinergic tone seen in the detrusor of patients with BOO due to BPH resembles findings obtained in females with OAB. Moreover, we showed here for the first time in a clinical setting that the higher urinary ATP levels observed in patients refractory to antimuscarinics fall significantly after their successful treatment with BoNT-A, suggesting that there is a close relationship between urinary ATP levels and symptoms severety. Our preliminary results also revealed that urinary ATP measurements may have a prognostic factor concerning the clinical outcome of BoNT-A treatments that is worth to pursuit by increasing the number of patients enrolled in the study.

In summary, this work added novel information highlightening the role of purines (ATP and ADO) in the pathophysiology, diagnostic and therapeutic of LUTS that may be clinically useful to manage patients afflicted by lower urinary tract dysfunctions, which constitute a tremendous burden for a large number of aged people and,thus, for health care services.

CHAPTER 5
REFERENCES

Abarbanel, J., & Marcus, E. L. (2007). Impaired detrusor contractility in community-dwelling elderly presenting with lower urinary tract symptoms. *Urology,* **69**(3), 436-440. doi:10.1016/j.urology.2006.11.019

Abdulqawi, R., Dockry, R., Holt, K., Layton, G., McCarthy, B. G., Ford, A. P., & Smith, J. A. (2015). P2X3 receptor antagonist (AF-219) in refractory chronic cough: a randomised, double-blind, placebo-controlled phase 2 study. *Lancet,* **385**(9974), 1198-1205. doi:10.1016/S0140-6736(14)61255-1

Abrams, P., Cardozo, L., Fall, M., Griffiths, D., Rosier, P., Ulmsten, U., van Kerrebroeck, P. Victor, A. Wein, A., Standardisation Sub-committee of the International Continence, S. (2002). The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-committee of the International Continence Society. *Neurourol Urodyn,* **21**(2), 167-178.

Abrams, P. H., Farrar, D. J., Turner-Warwick, R. T., Whiteside, C. G., & Feneley, R. C. (1979). The results of prostatectomy: a symptomatic and urodynamic analysis of 152 patients. *J Urol,* **121**(5), 640-642.

Acevedo, C. G., Contreras, E., Escalona, J., Lewin, J., & Huidobro-Toro, J. P. (1992). Pharmacological characterization of adenosine A1 and A2 receptors in the bladder: evidence for a modulatory adenosine tone regulating non-adrenergic non-cholinergic neurotransmission. *Br J Pharmacol,* **107**(1), 120-126.

Afeli, S. A., Hristov, K. L., & Petkov, G. V. (2012). Do beta3-adrenergic receptors play a role in guinea pig detrusor smooth muscle excitability and contractility? *Am J Physiol Renal Physiol,* **302**(2), F251-263. doi:10.1152/ajprenal.00378.2011

Agarwal, M. M., Patil, S., Roy, K., Bandawar, M., Choudhury, S., Mavuduru, R., Sharma, S. K., Mandal, A. K., Singh, S. K. (2014). Rationalization of interpretation of uroflowmetry for a non-caucasian (Indian) population: conceptual development and validation of volume-normalized flow rate index. *Neurourol Urodyn,* **33**(1), 135-141. doi:10.1002/nau.22388

Agarwal, R. P., Spector, T., & Parks, R. E., Jr. (1977). Tight-binding inhibitors--IV. Inhibition of adenosine deaminases by various inhibitors. *Biochem Pharmacol,* **26**(5), 359-367. doi:0006-2952(77)90192-7 [pii]

Aizawa, N., Igawa, Y., Andersson, K. E., Iijima, K., Nishizawa, O., & Wyndaele, J. J. (2011). Effects of intravesical instillation of ATP on rat bladder primary afferent activity and its relationship with capsaicin-sensitivity. *Neurourol Urodyn,* **30**(1), 163-168. doi:10.1002/nau.20940

Alexander, S. P., Benson, H. E., Faccenda, E., Pawson, A. J., Sharman, J. L., Spedding, M. Peters, J. A., Harmar, A. J., Cgtp Collaborators (2013a). The Concise Guide to PHARMACOLOGY 2013/14: G protein-coupled receptors. *Br J Pharmacol,* **170**(8), 1459-1581. doi:10.1111/bph.12445

Alexander, S. P., Benson, H. E., Faccenda, E., Pawson, A. J., Sharman, J. L., Spedding, M., Peters, J. A., Harmar, A. J., Cgtp Collaborators (2013b). The Concise Guide to PHARMACOLOGY 2013/14: ligand-gated ion channels. *Br J Pharmacol,* **170**(8), 1582-1606. doi:10.1111/bph.12446

Alkis, O., Zumrutbas, A. E., Toktas, C., Aybek, H., & Aybek, Z. (2015). The use of biomarkers in the diagnosis and treatment of overactive bladder: Can

we predict the patients who will be resistant to treatment? *Neurourol Urodyn*. doi:10.1002/nau.22939

Andersen, J. T., & Nordling, J. (1980). Prostatism. II. The correlation between cysto-urethroscopic, cystometric and urodynamic findings. *Scand J Urol Nephrol,* **14**(1), 23-27. doi:10.3109/00365598009181185

Andersson, K. E. (2003). Storage and voiding symptoms: pathophysiologic aspects. *Urology,* **62**(5 Suppl 2), 3-10. doi:S0090429503010495 [pii]

Antunes-Lopes, T., Carvalho-Barros, S., Cruz, C. D., Cruz, F., & Martins-Silva, C. (2011). Biomarkers in overactive bladder: a new objective and noninvasive tool? *Adv Urol, 2011*, 382431. doi:10.1155/2011/382431

Antunes-Lopes, T., Pinto, R., Barros, S. C., Botelho, F., Silva, C. M., Cruz, C. D., & Cruz, F. (2013). Urinary neurotrophic factors in healthy individuals and patients with overactive bladder. *J Urol,* **189**(1), 359-365. doi:10.1016/j.juro.2012.08.187

Apostolidis, A., de Nunzio, C., & Tubaro, A. (2012). What determines whether a patient with LUTS seeks treatment? ICI-RS 2011. *Neurourol Urodyn,* **31**(3), 365-369. doi:10.1002/nau.22212

Apostolidis, A., Popat, R., Yiangou, Y., Cockayne, D., Ford, A. P., Davis, J. B., . Dagupta P., Fowler C.J., Anand, P. (2005). Decreased sensory receptors P2X3 and TRPV1 in suburothelial nerve fibers following intradetrusor injections of botulinum toxin for human detrusor overactivity. *J Urol,* **174**(3), 977-982; discussion 982-973. doi:S0022-5347(01)68477-5 [pii] 10.1097/01.ju.0000169481.42259.54

Araki, I., Du, S., Kamiyama, M., Mikami, Y., Matsushita, K., Komuro, M., Furuya, Y. Takeda, M. (2004). Overexpression of epithelial sodium channels in epithelium of human urinary bladder with outlet obstruction. *Urology,* **64**(6), 1255-1260. doi:10.1016/j.urology.2004.06.064

Arch, J. R., & Newsholme, E. A. (1978a). Activities and some properties of 5'-nucleotidase, adenosine kinase and adenosine deaminase in tissues from vertebrates and invertebrates in relation to the control of the concentration and the physiological role of adenosine. *Biochem J,* **174**(3), 965-977.

Arch, J. R., & Newsholme, E. A. (1978b). The control of the metabolism and the hormonal role of adenosine. *Essays Biochem, 14*, 82-123.

Aronsson, P., Andersson, M., Ericsson, T., & Giglio, D. (2010). Assessment and characterization of purinergic contractions and relaxations in the rat urinary bladder. *Basic Clin Pharmacol Toxicol,* **107**(1), 603-613. doi:PTO554 [pii] 10.1111/j.1742-7843.2010.00554.x

Austin, J. C., Chacko, S. K., DiSanto, M., Canning, D. A., & Zderic, S. A. (2004). A male murine model of partial bladder outlet obstruction reveals changes in detrusor morphology, contractility and Myosin isoform expression. *J Urol,* **172(**4 Pt 1), 1524-1528.

Azadzoi, K. M., Pontari, M., Vlachiotis, J., & Siroky, M. B. (1996). Canine bladder blood flow and oxygenation: changes induced by filling, contraction and outlet obstruction. *J Urol,* **155**(4), 1459-1465. doi:S0022-5347(01)66307-9 [pii]

Banks, F. C., Knight, G. E., Calvert, R. C., Morgan, R. J., & Burnstock, G. (2006). Alterations in purinergic and cholinergic components of contractile responses of isolated detrusor contraction in a rat model of partial bladder outlet obstruction. *BJU Int,* **97**(2), 372-378. doi:BJU6010 [pii] 10.1111/j.1464-410X.2006.06010.x

Bates, C. P., Whiteside, C. G., & Turner-Warwick, R. (1970). Synchronous cine-pressure-flow-cysto-urethrography with special reference to stress and urge incontinence. *Br J Urol,* **42**(6), 714-723.

Bayliss, M., Wu, C., Newgreen, D., Mundy, A. R., & Fry, C. H. (1999). A quantitative study of atropine-resistant contractile responses in human detrusor smooth muscle, from stable, unstable and obstructed bladders. *J Urol,* **162**(5), 1833-1839. doi:S0022-5347(05)68247-X [pii]

Beckel, J. M., Daugherty, S. L., Tyagi, P., Wolf-Johnston, A. S., Birder, L. A., Mitchell, C. H., & de Groat, W. C. (2015). Pannexin 1 channels mediate the release of ATP into the lumen of the rat urinary bladder. *J Physiol,* **593**(8), 1857-1871. doi:10.1113/jphysiol.2014.283119

Belenky, A., Abarbanel, Y., Cohen, M., Yossepowitch, O., Livne, P. M., & Bachar, G. N. (2003). Detrusor resistive index evaluated by Doppler ultrasonography as a potential indicator of bladder outlet obstruction. *Urology,* **62**(4), 647-650.

Bhide, A. A., Cartwright, R., Khullar, V., & Digesu, G. A. (2013). Biomarkers in overactive bladder. *Int Urogynecol J,* **24**(7), 1065-1072. doi:10.1007/s00192-012-2027-1

Birder, L., & Andersson, K. E. (2013). Urothelial signaling. *Physiol Rev,* **93**(2), 653-680. doi:93/2/653 [pii] 10.1152/physrev.00030.2012

Birder, L. A. (2006). Urinary bladder urothelium: molecular sensors of chemical/thermal/mechanical stimuli. *Vascul Pharmacol,* **45**(4), 221-226. doi:S1537-1891(06)00155-8 [pii] 10.1016/j.vph.2005.08.027

Birder, L. A., Kanai, A. J., Cruz, F., Moore, K., & Fry, C. H. (2010). Is the urothelium intelligent? *Neurourol Urodyn,* **29**(4), 598-602. doi:10.1002/nau.20914

Birder, L. A., Ruan, H. Z., Chopra, B., Xiang, Z., Barrick, S., Buffington, C. A., Roppolo. J.R., Ford, A.P., de Groat, W., Burnstock, G. (2004). Alterations in P2X and P2Y purinergic receptor expression in urinary bladder from normal cats and cats with interstitial cystitis. *Am J Physiol Renal Physiol,* **287**(5), F1084-1091. doi:10.1152/ajprenal.00118.2004 00118.2004 [pii]

Bodin, P., & Burnstock, G. (1996). ATP-stimulated release of ATP by human endothelial cells. *J Cardiovasc Pharmacol,* **27**(6), 872-875.

Boyle, P., Robertson, C., Mazzetta, C., Keech, M., Hobbs, F. D., Fourcade, R., Kiemeney, L., Lee, C., UrEpik Study Group (2003). The prevalence of lower urinary tract symptoms in men and women in four centres. The UrEpik study. *BJU Int,* **92**(4), 409-414.

Brading, A. F., & Williams, J. H. (1990). Contractile responses of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation: effects of atropine and alpha,beta-methylene ATP. *Br J Pharmacol,* **99**(3), 493-498.

Brady, C. M., Apostolidis, A., Yiangou, Y., Baecker, P. A., Ford, A. P., Freeman, A., Jacques, T.S., Fowler, C.J., Anand, P. (2004). P2X3-immunoreactive nerve fibres in neurogenic detrusor overactivity and the effect of intravesical resiniferatoxin. *Eur Urol,* **46**(2), 247-253. doi:10.1016/j.eururo.2003.12.017

Braverman, A. S., & Ruggieri, M. R., Sr. (2003). Hypertrophy changes the muscarinic receptor subtype mediating bladder contraction from M3 toward M2. *Am J Physiol Regul Integr Comp Physiol,* **285**(3), R701-708. doi:10.1152/ajpregu.00009.2003

Brostrom, S., Jennum, P., & Lose, G. (2002). Short-term reproducibility of cystometry and pressure-flow micturition studies in healthy women. *Neurourol Urodyn,* **21**(5), 457-460. doi:10.1002/nau.10019

Brown, C., Burnstock, G., & Cocks, T. (1979). Effects of adenosine 5'-triphosphate (ATP) and beta-gamma-methylene ATP on the rat urinary bladder. *Br J Pharmacol,* **65**(1), 97-102.

Brown, J. S., Vittinghoff, E., Wyman, J. F., Stone, K. L., Nevitt, M. C., Ensrud, K. E., & Grady, D. (2000). Urinary incontinence: does it increase risk for falls and fractures? Study of Osteoporotic Fractures Research Group. *J Am Geriatr Soc,* **48**(7), 721-725.

Brown, S. M., Bentcheva-Petkova, L. M., Liu, L., Hristov, K. L., Chen, M., Kellett, W. F., Meredith, A. L., Aldrich, R. W., Nelson, M. T., Petkov, G. V. (2008). Beta-adrenergic relaxation of mouse urinary bladder smooth muscle in the absence of large-conductance Ca2+-activated K+ channel. *Am J Physiol Renal Physiol,* **295**(4), F1149-1157. doi:10.1152/ajprenal.00440.2007

Burnstock, G. (1972). Purinergic nerves. *Pharmacol Rev,* **24**(3), 509-581.

Burnstock, G. (2000). P2X receptors in sensory neurones. *Br J Anaesth,* **84**(4), 476-488.

Burnstock, G. (2006). Purinergic signalling. *Br J Pharmacol,* **147** *Suppl 1*, S172-181. doi:0706429 [pii] 10.1038/sj.bjp.0706429

Burnstock, G. (2011). Therapeutic potential of purinergic signalling for diseases of the urinary tract. *BJU Int,* **107**(2), 192-204. doi:10.1111/j.1464-410X.2010.09926.x

Burnstock, G. (2014). Purinergic signalling in the urinary tract in health and disease. *Purinergic Signal,* **10**(1), 103-155. doi:10.1007/s11302-013-9395-y

Burnstock, G., Cocks, T., Crowe, R., & Kasakov, L. (1978a). Purinergic innervation of the guinea-pig urinary bladder. *Br J Pharmacol,* **63**(1), 125-138.

Burnstock, G., Cocks, T., Kasakov, L., & Wong, H. K. (1978b). Direct evidence for ATP release from non-adrenergic, non-cholinergic ("purinergic") nerves in the guinea-pig taenia coli and bladder. *Eur J Pharmacol,* **49**(2), 145-149. doi:0014-2999(78)90070-5 [pii]

Burnstock, G., Dumsday, B., & Smythe, A. (1972). Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *Br J Pharmacol,* **44**(3), 451-461.

Burnstock, G., & Verkhratsky, A. (2010). Long-term (trophic) purinergic signalling: purinoceptors control cell proliferation, differentiation and death. *Cell Death Dis,* **1**, e9. doi:10.1038/cddis.2009.11

Carneiro, I., Timóteo, M. A., Silva, I., Vieira, C., Baldaia, C., Ferreirinha, F., Silva-Ramos, M., Correia-de-Sá, P. (2014). Activation of P2Y6 receptors increases the voiding frequency in anaesthetized rats by releasing ATP from the bladder urothelium. *Br J Pharmacol,* **171**(14), 3404-3419. doi:10.1111/bph.12711

Cartwright, R., Afshan, I., Derpapas, A., Vijaya, G., & Khullar, V. (2011). Novel biomarkers for overactive bladder. *Nat Rev Urol,* **8**(3), 139-145. doi:nrurol.2011.7 [pii] 10.1038/nrurol.2011.7

Castren, E., & Rantamaki, T. (2010). Role of brain-derived neurotrophic factor in the aetiology of depression: implications for pharmacological treatment. *CNS Drugs,* **24**(1), 1-7. doi:10.2165/11530010-000000000-00000

Chai, T. C., Gray, M. L., & Steers, W. D. (1998). The incidence of a positive ice water test in bladder outlet obstructed patients: evidence for bladder neural plasticity. *J Urol,* **160**(1), 34-38.

Chapple, C. R., Milner, P., Moss, H. E., & Burnstock, G. (1992). Loss of sensory neuropeptides in the obstructed human bladder. *Br J Urol,* **70**(4), 373-381.

Cheng, Y., Mansfield, K. J., Allen, W., Chess-Williams, R., Burcher, E., & Moore, K. H. (2014). ATP during early bladder stretch is important for urgency in detrusor overactivity patients. *Biomed Res Int, 2014*, 204604. doi:10.1155/2014/204604

Cheng, Y., Mansfield, K. J., Allen, W., Millard, R. J., Burcher, E., & Moore, K. H. (2013). Correlation between cystometric volumes, ATP release, and pH in women with overactive bladder versus controls. *Neurourol Urodyn,* **32**(7), 969-973. doi:10.1002/nau.22344

Cheng, Y., Mansfield, K. J., Allen, W., Walsh, C. A., Burcher, E., & Moore, K. H. (2010). Does adenosine triphosphate released into voided urodynamic fluid contribute to urgency signaling in women with bladder dysfunction? *J Urol,* **183**(3), 1082-1086. doi:S0022-5347(09)02917-6 [pii] 10.1016/j.juro.2009.11.028

Chopra, B., Gever, J., Barrick, S. R., Hanna-Mitchell, A. T., Beckel, J. M., Ford, A. P., & Birder, L. A. (2008). Expression and function of rat urothelial P2Y receptors. *Am J Physiol Renal Physiol,* **294**(4), F821-829. doi:10.1152/ajprenal.00321.2006

Chua, W. C., Liu, L., Mansfield, K. J., Vaux, K. J., Moore, K. H., Millard, R. J., & Burcher, E. (2007). Age-related changes of P2X(1) receptor mRNA in the bladder detrusor from men with and without bladder outlet obstruction. *Exp Gerontol,* **42**(7), 686-692. doi:S0531-5565(07)00034-4 [pii] 10.1016/j.exger.2007.02.003

Chuang, Y. C., Yoshimura, N., Huang, C. C., Chiang, P. H., & Chancellor, M. B. (2004). Intravesical botulinum toxin a administration produces analgesia against acetic acid induced bladder pain responses in rats. *J Urol,* **172**(4 Pt 1), 1529-1532.

Cockayne, D. A., Dunn, P. M., Zhong, Y., Rong, W., Hamilton, S. G., Knight, G. E., Ruan, H. Z., Ma, B., Yip, P., Nunn, P., McMahon, S. B., Burnstock, G., Ford, A. P. (2005). P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP. *J Physiol,* **567**(Pt 2), 621-639. doi:jphysiol.2005.088435 [pii] 10.1113/jphysiol.2005.088435

Cockayne, D. A., Hamilton, S. G., Zhu, Q. M., Dunn, P. M., Zhong, Y., Novakovic, S., Malmberg, A. B., Cain, G., Berson, A., Kassotakis, L., Hedley, L., Lachnit, W. G., Burnstock, G., McMahon, S. B., Ford, A. P. (2000). Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature,* **407**(6807), 1011-1015. doi:10.1038/35039519

Cohen, M. V., & Downey, J. M. (2008). Adenosine: trigger and mediator of cardioprotection. *Basic Res Cardiol,* **103**(3), 203-215. doi:10.1007/s00395-007-0687-7

Collins, V. M., Daly, D. M., Liaskos, M., McKay, N. G., Sellers, D., Chapple, C., & Grundy, D. (2013). OnabotulinumtoxinA significantly attenuates bladder afferent nerve firing and inhibits ATP release from the urothelium. *BJU Int,* **112**(7), 1018-1026. doi:10.1111/bju.12266

Correia-de-Sá, P. (2010). Pathophysiological significance of nucleotide hydrolyzing enzymes in urologic disorder. *Purinergic Signal,* **6**(Suppl 2), S13.13.

Correia-de-Sá, P., Adaes, S., Timóteo, M. A., Vieira, C., Magalhães-Cardoso, T., Nascimento, C., & Duarte-Araújo, M. (2006). Fine-tuning modulation of myenteric motoneurons by endogenous adenosine: on the role of secreted adenosine deaminase. *Auton Neurosci,* **126-127**, 211-224. doi:S1566-0702(06)00052-X [pii] 10.1016/j.autneu.2006.02.004

Correia-de-Sá, P., & Ribeiro, J. A. (1996). Adenosine uptake and deamination regulate tonic A2a receptor facilitation of evoked [3H]acetylcholine release from the rat motor nerve terminals. *Neuroscience,* ***73***(1), 85-92. doi:0306452296000280 [pii]

Correia-de-Sá, P., Sebastião, A. M., & Ribeiro, J. A. (1991). Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve ending of the rat. *Br J Pharmacol,* **103**(2), 1614-1620.

Cowan, W. D., & Daniel, E. E. (1983). Human female bladder and its noncholinergic contractile function. *Can J Physiol Pharmacol,* **61**(11), 1236-1246.

Coyne, K. S., Sexton, C. C., Irwin, D. E., Kopp, Z. S., Kelleher, C. J., & Milsom, I. (2008). The impact of overactive bladder, incontinence and other lower urinary tract symptoms on quality of life, work productivity, sexuality and emotional well-being in men and women: results from the EPIC study. *BJU Int,* **101**(11), 1388-1395. doi:10.1111/j.1464-410X.2008.07601.x

Coyne, K. S., Sexton, C. C., Kopp, Z. S., Ebel-Bitoun, C., Milsom, I., & Chapple, C. (2011). The impact of overactive bladder on mental health, work productivity and health-related quality of life in the UK and Sweden: results from EpiLUTS. *BJU Int*. **108**(9),1459-71. doi:10.1111/j.1464-410X.2010.10013.x

Cruz, F. (2014). Targets for botulinum toxin in the lower urinary tract. *Neurourol Urodyn,* **33**(1), 31-38. doi:10.1002/nau.22445

Cruz, F., Herschorn, S., Aliotta, P., Brin, M., Thompson, C., Lam, W., Daniel, G., Heesakkers, J., Haag-Molkenteller, C. (2011). Efficacy and safety of onabotulinumtoxinA in patients with urinary incontinence due to neurogenic detrusor overactivity: a randomised, double-blind, placebo-controlled trial. *Eur Urol,* **60**(4), 742-750. doi:10.1016/j.eururo.2011.07.002

Cusack, N. J., & Hourani, S. M. (1984). Some pharmacological and biochemical interactions of the enantiomers of adenylyl 5'-(beta, gamma-methylene)-diphosphonate with the guinea-pig urinary bladder. *Br J Pharmacol,* **82**(1), 155-159.

D'Agostino, G., Condino, A. M., Calvi, V., Boschi, F., Gioglio, L., & Barbieri, A. (2012). Purinergic P2X3 heteroreceptors enhance parasympathetic motor drive in isolated porcine detrusor, a reliable model for development of P2X selective blockers for detrusor hyperactivity. *Pharmacol Res,* **65(**1), 129-136. doi:S1043-6618(11)00276-3 [pii] 10.1016/j.phrs.2011.10.002

D'Silva, K. A., Dahm, P., & Wong, C. L. (2014). Does this man with lower urinary tract symptoms have bladder outlet obstruction?: The Rational Clinical Examination: a systematic review. *JAMA,* **312**(5), 535-542. doi:10.1001/jama.2014.5555

de la Rosette, J. J., Witjes, W. P., Schafer, W., Abrams, P., Donovan, J. L., Peters, T. J., Millard, R.J., Frimodt-Moller, C., Kalomiris, P. (1998). Relationships between lower urinary tract symptoms and bladder outlet obstruction: results from the ICS-"BPH" study. *Neurourol Urodyn,* **17**(2), 99-108.

de Nunzio, C., Franco, G., Rocchegiani, A., Iori, F., Leonardo, C., & Laurenti, C. (2003). The evolution of detrusor overactivity after watchful waiting, medical therapy and surgery in patients with bladder outlet obstruction. *J Urol,* **169**(2), 535-539. doi:10.1097/01.ju.0000045600.69261.73

Dean, D. M., & Downie, J. W. (1978). Contribution of adrenergic and "purinergic" neurotransmission to contraction in rabbit detrusor. *J Pharmacol Exp Ther,* **207**(2), 431-445.

Denys, P., Le Normand, L., Ghout, I., Costa, P., Chartier-Kastler, E., Grise, P., Hermieu, J. F., Amarenco, G., Karsenty, G., Saussine, C., Barbot, F., Vesitox study group in France (2012). Efficacy and safety of low doses of onabotulinumtoxinA for the treatment of refractory idiopathic overactive bladder: a multicentre, double-blind, randomised, placebo-controlled dose-ranging study. *Eur Urol,* **61**(3), 520-529. doi:10.1016/j.eururo.2011.10.028

Dinis, P., Silva, J., Ribeiro, M. J., Avelino, A., Reis, M., & Cruz, F. (2004). Bladder C-fiber desensitization induces a long-lasting improvement of BPH-associated storage LUTS: a pilot study. *Eur Urol,* **46**(1), 88-93; discussion 93-84. doi:10.1016/j.eururo.2004.01.016

Dixon, A. K., Gubitz, A. K., Sirinathsinghji, D. J., Richardson, P. J., & Freeman, T. C. (1996). Tissue distribution of adenosine receptor mRNAs in the rat. *Br J Pharmacol,* **118**(6), 1461-1468.

Duarte-Araújo, M., Nascimento, C., Alexandrina Timoteo, M., Magalhães-Cardoso, T., & Correia-de-Sá, P. (2004). Dual effects of adenosine on acetylcholine release from myenteric motoneurons are mediated by junctional facilitatory A(2A) and extrajunctional inhibitory A(1) receptors. *Br J Pharmacol,* **141**(6), 925-934. doi:10.1038/sj.bjp.0705697 sj.bjp.0705697 [pii]

Duarte-Araújo, M., Nascimento, C., Timoteo, M. A., Magalhães-Cardoso, M. T., & Correia-de-Sá, P. (2009). Relative contribution of ecto-ATPase and ecto-ATPDase pathways to the biphasic effect of ATP on acetylcholine release from myenteric motoneurons. *Br J Pharmacol,* **156**(3), 519-533. doi:BPH058 [pii] 10.1111/j.1476-5381.2008.00058.x

DuBeau, C. E., Kiely, D. K., & Resnick, N. M. (1999). Quality of life impact of urge incontinence in older persons: a new measure and conceptual structure. *J Am Geriatr Soc,* **47**(8), 989-994.

Dugan, E., Cohen, S. J., Bland, D. R., Preisser, J. S., Davis, C. C., Suggs, P. K., & McGann, P. (2000). The association of depressive symptoms and urinary incontinence among older adults. *J Am Geriatr Soc,* **48**(4), 413-416.

Dunn, P. M., Zhong, Y., & Burnstock, G. (2001). P2X receptors in peripheral neurons. *Prog Neurobiol,* **65**(2), 107-134.

Dunning-Davies, B. M., Fry, C. H., Mansour, D., & Ferguson, D. R. (2013). The regulation of ATP release from the urothelium by adenosine and transepithelial potential. *BJU Int,* **111**(3), 505-513. doi:10.1111/j.1464-410X.2012.11421.x

Dunwiddie, T. V., & Masino, S. A. (2001). The role and regulation of adenosine in the central nervous system. *Annu Rev Neurosci,* **24**, 31-55. doi:10.1146/annurev.neuro.24.1.31 24/1/31 [pii]

Eckhardt, M. D., van Venrooij, G. E., & Boon, T. A. (2001). Symptoms and quality of life versus age, prostate volume, and urodynamic parameters in 565 strictly selected men with lower urinary tract symptoms suggestive of benign prostatic hyperplasia. *Urology,* **57(**4), 695-700.

Egan, J. P. (1975). *Signal detection theory and ROC analysis.* New York: Academic Press.

Elliott, R. A., Tonnu, A., Ghaffar, N., Taylor, A. H., Tincello, D. G., & Norman, R. I. (2013). Enhanced purinergic contractile responses and P2X1 receptor expression in detrusor muscle during cycles of hypoxia-glucopenia and reoxygenation. *Exp Physiol,* **98**(12), 1683-1695. doi:expphysiol.2013.075010 [pii] 10.1113/expphysiol.2013.075010

Elneil, S., Skepper, J. N., Kidd, E. J., Williamson, J. G., & Ferguson, D. R. (2001). Distribution of P2X(1) and P2X(3) receptors in the rat and human urinary bladder. *Pharmacology,* **63**(2), 120-128. doi:56122

Elterman, D. S., Chughtai, B., Lee, R., Te, A. E., & Kaplan, S. A. (2013). Noninvasive methods to evaluate bladder obstruction in men. *Int Braz J Urol,* **39**(1), 4-9. doi:10.1590/S1677-5538.IBJU.2013.01.02

Engstrom, G., Henningsohn, L., Steineck, G., & Leppert, J. (2005). Self-assessed health, sadness and happiness in relation to the total burden of symptoms from the lower urinary tract. *BJU Int,* **95**(6), 810-815. doi:10.1111/j.1464-410X.2005.05406.x

Farag, F. F., & Heesakkers, J. P. (2011). Non-invasive techniques in the diagnosis of bladder storage disorders. *Neurourol Urodyn,* **30**(8), 1422-1428. doi:10.1002/nau.21155

Ferguson, D. R., Kennedy, I., & Burton, T. J. (1997). ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes--a possible sensory mechanism? *J Physiol,* **505** *(Pt 2)*, 503-511.

Ford, A. P., & Cockayne, D. A. (2011). ATP and P2X purinoceptors in urinary tract disorders. *Handb Exp Pharmacol* (202), 485-526. doi:10.1007/978-3-642-16499-6_22

Ford, A. P., Gever, J. R., Nunn, P. A., Zhong, Y., Cefalu, J. S., Dillon, M. P., & Cockayne, D. A. (2006). Purinoceptors as therapeutic targets for lower urinary tract dysfunction. *Br J Pharmacol,* **147** *Suppl 2*, S132-143. doi:0706637 [pii] 10.1038/sj.bjp.0706637

Ford, A. P., & Undem, B. J. (2013). The therapeutic promise of ATP antagonism at P2X3 receptors in respiratory and urological disorders. *Front Cell Neurosci,* **7**, 267. doi:10.3389/fncel.2013.00267

Fredholm, B. B., AP, I. J., Jacobson, K. A., Klotz, K. N., & Linden, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev,* **53**(4), 527-552.

Fredholm, B. B., AP, I. J., Jacobson, K. A., Linden, J., & Muller, C. E. (2011). International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update. *Pharmacol Rev,* **63**(1), 1-34. doi:10.1124/pr.110.003285

Fry, C. H., Sahai, A., Vahabi, B., Kanai, A. J., & Birder, L. A. (2014). What is the role for biomarkers for lower urinary tract disorders? ICI-RS 2013. *Neurourol Urodyn,* **33**(5), 602-605. doi:10.1002/nau.22558

Fry, C. H., Sui, G. P., Kanai, A. J., & Wu, C. (2007). The function of suburothelial myofibroblasts in the bladder. *Neurourol Urodyn,* **26**(6 Suppl), 914-919. doi:10.1002/nau.20483

Fry, C. H., Wu, C., & Mundy, A. R. (1999). Bladder instability and detrusor smooth muscle function. *Exp Physiol,* **84**(1), 161-169.

Fry, C. H., Young, J. S., Jabr, R. I., McCarthy, C., Ikeda, Y., & Kanai, A. J. (2012). Modulation of spontaneous activity in the overactive bladder: the role of P2Y agonists. *Am J Physiol Renal Physiol,* **302**(11), F1447-1454. doi:10.1152/ajprenal.00436.2011

Gammie, A., Kaper, M., Dorrepaal, C., Kos, T., & Abrams, P. (2015). Signs and Symptoms of Detrusor Underactivity: An Analysis of Clinical Presentation and Urodynamic Tests From a Large Group of Patients Undergoing Pressure Flow Studies. *Eur Urol*, **69**(2), 361-9. doi:10.1016/j.eururo.2015.08.014

Geiger, J. F., DM. (1991). *Adenosine in the Nervous System: Adenosine transport in nervous system tissues* (Unitated States Edition ed.): San Diego: Academic Press Inc.

Geloso, D. A., & Levin, R. M. (1998). Effect of partial outlet obstruction on the myogenic response to field stimulation. *Gen Pharmacol,* **31**(2), 291-295.

Gever, J. R., Soto, R., Henningsen, R. A., Martin, R. S., Hackos, D. H., Panicker, S., . . . Ford, A. P. (2010). AF-353, a novel, potent and orally bioavailable P2X3/P2X2/3 receptor antagonist. *Br J Pharmacol,* **160**(6), 1387-1398. doi:BPH796 [pii] 10.1111/j.1476-5381.2010.00796.x

Gill, K., Horsley, H., Kupelian, A. S., Baio, G., De Iorio, M., Sathiananamoorthy, S., . . . Malone-Lee, J. (2015). Urinary ATP as an indicator of infection and inflammation of the urinary tract in patients with lower urinary tract symptoms. *BMC Urol,* **15**, 7. doi:10.1186/s12894-015-0001-1

Gonçalves, J., & Queiroz, G. (1993). Facilitatory and inhibitory modulation by endogenous adenosine of noradrenaline release in the epididymal portion of rat vas deferens. *Naunyn Schmiedebergs Arch Pharmacol,* **348**(4), 367-371.

Gopalakrishnan, S. M., Buckner, S. A., Milicic, I., Groebe, D. R., Whiteaker, K. L., Burns, D. J., Warrior, U., Gopalakrishnan, M. (2002). Functional characterization of adenosine receptors and coupling to ATP-sensitive K+ channels in Guinea pig urinary bladder smooth muscle. *J Pharmacol Exp Ther,* **300**(3), 910-917.

Gosling, J. A. (1997). Modification of bladder structure in response to outflow obstruction and ageing. *Eur Urol,* **32** *Suppl 1*, 9-14.

Gratzke, C., Bachmann, A., Descazeaud, A., Drake, M. J., Madersbacher, S., Mamoulakis, C., Oelke, M., Tikkinen, K.A., Gravas, S. (2015). EAU Guidelines on the Assessment of Non-neurogenic Male Lower Urinary Tract Symptoms including Benign Prostatic Obstruction. *Eur Urol,* **67**(6), 1099-1109. doi:10.1016/j.eururo.2014.12.038

Griffith, D. A., & Jarvis, S. M. (1996). Nucleoside and nucleobase transport systems of mammalian cells. *Biochim Biophys Acta,* **1286**(3), 153-181. doi:S0304-4157(96)00008-1 [pii]

Hampel, C., Dolber, P. C., Smith, M. P., Savic, S. L., Th roff, J. W., Thor, K. B., & Schwinn, D. A. (2002). Modulation of bladder alpha1-adrenergic receptor subtype expression by bladder outlet obstruction. *J Urol,* **167**(3), 1513-1521.

Hanley, J. A., & McNeil, B. J. (1983). A method of comparing the areas under receiver operating characteristic curves derived from the same cases. *Radiology,* **148**(3), 839-843. doi:10.1148/radiology.148.3.6878708

Hanna-Mitchell, A. T., Wolf-Johnston, A. S., Barrick, S. R., Kanai, A. J., Chancellor, M. B., de Groat, W. C., & Birder, L. A. (2015). Effect of botulinum toxin A on urothelial-release of ATP and expression of SNARE targets within the urothelium. *Neurourol Urodyn,* **34**(1), 79-84. doi:10.1002/nau.22508

Harrison, S. C., Hunnam, G. R., Farman, P., Ferguson, D. R., & Doyle, P. T. (1987). Bladder instability and denervation in patients with bladder outflow obstruction. *Br J Urol,* **60**(6), 519-522.

Harvey, R. A., Skennerton, D. E., Newgreen, D., & Fry, C. H. (2002). The contractile potency of adenosine triphosphate and ecto-adenosine triphosphatase activity in guinea pig detrusor and detrusor from patients with a stable, unstable or obstructed bladder. *J Urol,* **168**(3), 1235-1239. doi:10.1097/01.ju.0000024391.74486.f1 S0022-5347(05)64632-0 [pii]

Hashim, H., & Abrams, P. (2006). Is the bladder a reliable witness for predicting detrusor overactivity? *J Urol,* **175**(1), 191-194; discussion 194-195. doi:S0022-5347(05)00067-4 [pii] 10.1016/S0022-5347(05)00067-4

Hasko, G., Linden, J., Cronstein, B., & Pacher, P. (2008). Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov,* **7**(9), 759-770. doi:nrd2638 [pii] 10.1038/nrd2638

Hegde, S. S., Mandel, D. A., Wilford, M. R., Briaud, S., Ford, A. P., & Eglen, R. M. (1998). Evidence for purinergic neurotransmission in the urinary bladder of pithed rats. *Eur J Pharmacol,* **349**(1), 75-82.

Hernandez, M., Barahona, M. V., Bustamante, S., Garcia-Sacristan, A., & Orensanz, L. M. (1999). A2B adenosine receptors mediate relaxation of the pig intravesical ureter: adenosine modulation of non adrenergic non cholinergic excitatory neurotransmission. *Br J Pharmacol,* **126**(4), 969-978. doi:10.1038/sj.bjp.0702386

Hernandez, M., Knight, G. E., Wildman, S. S., & Burnstock, G. (2009). Role of ATP and related purines in inhibitory neurotransmission to the pig urinary bladder neck. *Br J Pharmacol, 157*(8), 1463-1473. doi:BPH314 [pii] 10.1111/j.1476-5381.2009.00314.x

Heyne, N., Benohr, P., Muhlbauer, B., Delabar, U., Risler, T., & Osswald, H. (2004). Regulation of renal adenosine excretion in humans--role of sodium and fluid homeostasis. *Nephrol Dial Transplant,* **19**(11), 2737-2741. doi:10.1093/ndt/gfh492 gfh492 [pii]

Hu, T. W., Wagner, T. H., Bentkover, J. D., LeBlanc, K., Piancentini, A., Stewart, W. F., Corey, R., Zhou, S. Z., Hunt, T. L. (2003). Estimated economic costs of overactive bladder in the United States. *Urology,* **61**(6), 1123-1128.

Husted, S., Sjogren, C., & Andersson, K. E. (1983). Direct effects of adenosine and adenine nucleotides on isolated human urinary bladder and their influence on electrically induced contractions. *J Urol,* **130**(2), 392-398.

Hypolite, J. A., Longhurst, P. A., Gong, C., Briscoe, J., Wein, A. J., & Levin, R. M. (1993). Metabolic studies on rabbit bladder smooth muscle and mucosa. *Mol Cell Biochem,* **125**(1), 35-42.

Igawa, Y., Mattiasson, A., & Andersson, K. E. (1993). Functional importance of cholinergic and purinergic neurotransmission for micturition contraction in the normal, unanaesthetized rat. *Br J Pharmacol,* **109**(2), 473-479.

Inui, E., Ochiai, A., Naya, Y., Ukimura, O., & Kojima, M. (1999). Comparative morphometric study of bladder detrusor between patients with benign prostatic hyperplasia and controls. *J Urol,* **161**(3), 827-830.

Irwin, D. E., Kopp, Z. S., Agatep, B., Milsom, I., & Abrams, P. (2011). Worldwide prevalence estimates of lower urinary tract symptoms, overactive bladder, urinary incontinence and bladder outlet obstruction. *BJU Int,* **108**(7), 1132-1138. doi:10.1111/j.1464-410X.2010.09993.x

Irwin, D. E., Milsom, I., Hunskaar, S., Reilly, K., Kopp, Z., Herschorn, S., Coyne, K., Kelleher, C., Hampel, C., Artibani, W., Abrams, P. (2006). Population-based survey of urinary incontinence, overactive bladder, and other lower urinary tract symptoms in five countries: results of the EPIC study. *Eur Urol,* **50**(6), 1306-1314; discussion 1314-1305. doi:S0302-2838(06)01116-X [pii] 10.1016/j.eururo.2006.09.019

Irwin, D. E., Milsom, I., Kopp, Z., Abrams, P., Artibani, W., & Herschorn, S. (2009). Prevalence, severity, and symptom bother of lower urinary tract symptoms among men in the EPIC study: impact of overactive bladder. *Eur Urol,* **56**(1), 14-20. doi: S0302-2838(09)00196-1[pii] 10.1016/j.eururo.2009.02.026

Ito, K., Iwami, A., Katsura, H., & Ikeda, M. (2008). Therapeutic effects of the putative P2X3/P2X2/3 antagonist A-317491 on cyclophosphamide-induced cystitis in rats. *Naunyn Schmiedebergs Arch Pharmacol,* **377**(4-6), 483-490. doi:10.1007/s00210-007-0197-z

Jackson, E. K., & Raghvendra, D. K. (2004). The extracellular cyclic AMP-adenosine pathway in renal physiology. *Annu Rev Physiol,* **66**, 571-599. doi:10.1146/annurev.physiol.66.032102.111604

Jackson, E. K., Ren, J., Cheng, D., & Mi, Z. (2011). Extracellular cAMP-adenosine pathways in the mouse kidney. *Am J Physiol Renal Physiol,* **301**(3), F565-573. doi:ajprenal.00094.2011 [pii] 10.1152/ajprenal.00094.2011

Jeong, S. J., Kim, H. J., Lee, Y. J., Lee, J. K., Lee, B. K., Choo, Y. M., Oh, J. J., Lee, S. C., Jeong, C. W., Yoon, C. Y., Hong, S. K., Byun, S. S., Lee, S. E. (2012). Prevalence and Clinical Features of Detrusor Underactivity among Elderly with Lower Urinary Tract Symptoms: A Comparison between Men and Women. *Korean J Urol,* **53**(5), 342-348. doi:10.4111/kju.2012.53.5.342

Juszczak, K., & Drewa, T. (2016). Pharmacotherapy in detrusor underactivity: A new challenge for urologists and pharmacologists (from lab to clinic). *Pharmacol Rep,* **68**(4), 703-706. doi:10.1016/j.pharep.2016.03.007

Kaelin-Lang, A., Lauterburg, T., & Burgunder, J. M. (1998). Expression of adenosine A2a receptor gene in rat dorsal root and autonomic ganglia. *Neurosci Lett,* **246**(1), 21-24. doi:S0304-3940(98)00216-X [pii]

Keiding, R. H., M.; Gerhardt Denmark, W.; Pitkänen, E; Tenhunen,R.; Strömme, J.H.; Liv Theodorsen; Waldenström, J.; Tryding, N , & Westlund L. (1974). Recommended methods for the determination of four enzymes in blood. *Scand J Clin Lab Invest,* **33**(4), 291-306. doi:10.1080/00365517409082499

Khakh, B. S., & North, R. A. (2012). Neuromodulation by extracellular ATP and P2X receptors in the CNS. *Neuron,* **76**(1), 51-69. doi:10.1016/j.neuron.2012.09.024

Khera, M., Somogyi, G. T., Kiss, S., Boone, T. B., & Smith, C. P. (2004). Botulinum toxin A inhibits ATP release from bladder urothelium after chronic spinal cord injury. *Neurochem Int,* **45**(7), 987-993. doi:10.1016/j.neuint.2004.06.001 S0197018604001111 [pii]

Kim, J. C., Park, E. Y., Seo, S. I., Park, Y. H., & Hwang, T. K. (2006). Nerve growth factor and prostaglandins in the urine of female patients with overactive bladder. *J Urol,* **175**(5), 1773-1776; discussion 1776. doi:S0022-5347(05)00992-4 [pii] 10.1016/S0022-5347(05)00992-4

Kim, S. O., Oh, B. S., Chang, I. Y., Song, S. H., Ahn, K., Hwang, E. C., Oh, K.J:, Kwon, D., Park, K. (2011). Distribution of interstitial cells of Cajal and expression of nitric oxide synthase after experimental bladder outlet obstruction in a rat model of bladder overactivity. *Neurourol Urodyn,* **30**(8), 1639-1645. doi:10.1002/nau.21144

King, B. F., Knowles, I. D., Burnstock, G., & Ramage, A. G. (2004). Investigation of the effects of P2 purinoceptor ligands on the micturition reflex in female urethane-anaesthetized rats. *Br J Pharmacol,* **142**(3), 519-530. doi:10.1038/sj.bjp.0705790 0705790 [pii]

King, J. A., Huddart, H., & Staff, W. G. (1997). Purinergic modulation of rat urinary bladder detrusor smooth muscle. *Gen Pharmacol,* **29**(4), 597-604. doi:S0306362396005733 [pii]

Kitta, T., Chancellor, M. B., de Groat, W. C., Kuno, S., Nonomura, K., & Yoshimura, N. (2012). Suppression of bladder overactivity by adenosine A2A receptor antagonist in a rat model of Parkinson disease. *J Urol,* **187**(5), 1890-1897. doi:S0022-5347(11)06009-5 [pii] 10.1016/j.juro.2011.12.062

Kitta, T., Chancellor, M. B., de Groat, W. C., Kuno, S., Nonomura, K., & Yoshimura, N. (2014). Roles of adenosine A1 and A2A receptors in the control of micturition in rats. *Neurourol Urodyn,* **33**(8), 1259-1265. doi:10.1002/nau.22487

Klinger, M., Freissmuth, M., & Nanoff, C. (2002). Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. *Cell Signal,* **14**(2), 99-108. doi:S0898656801002352 [pii]

Kojima, M., Inui, E., Ochiai, A., Naya, Y., Ukimura, O., & Watanabe, H. (1996). Ultrasonic estimation of bladder weight as a measure of bladder hypertrophy in men with infravesical obstruction: a preliminary report. *Urology,* **47**(6), 942-947. doi:10.1016/S0090-4295(96)00059-3

Kosan, M., Tul, M., Ozturk, B., Hafez, G., Inal, G., & Cetinkaya, M. (2008). Alteration in contractile responses in human detrusor smooth muscle from obstructed bladders with overactivity. *Urol Int,* **80**(2), 193-200. doi:000112613 [pii] 10.1159/000112613

Kukulski, F., Levesque, S. A., Lavoie, E. G., Lecka, J., Bigonnesse, F., Knowles, A. F., Robson, S.C., Kirley, T.L., Sevigny, J. (2005). Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. *Purinergic Signal,* **1**(2), 193-204. doi:10.1007/s11302-005-6217-x

Kullmann, F. A., Artim, D., Beckel, J., Barrick, S., de Groat, W. C., & Birder, L. A. (2008). Heterogeneity of muscarinic receptor-mediated Ca2+ responses in cultured urothelial cells from rat. *Am J Physiol Renal Physiol,* **294**(4), F971-981. doi:00313.2007 [pii] 10.1152/ajprenal.00313.2007

Kumar, V., Chapple, C. C., & Chess-Williams, R. (2004). Characteristics of adenosine triphosphate [corrected] release from porcine and human

normal bladder. *J Urol,* **172**(2), 744-747. doi:00005392-200408000-00082 [pii]

Kumar, V., Chapple, C. R., Rosario, D., Tophill, P. R., & Chess-Williams, R. (2010). In vitro release of adenosine triphosphate from the urothelium of human bladders with detrusor overactivity, both neurogenic and idiopathic. *Eur Urol,* **57**(6), 1087-1092. doi:S0302-2838(09)01204-4 [pii] 10.1016/j.eururo.2009.11.042

Kumar, V., Chapple, C. R., Surprenant, A. M., & Chess-Williams, R. (2007). Enhanced adenosine triphosphate release from the urothelium of patients with painful bladder syndrome: a possible pathophysiological explanation. *J Urol,* **178**(4 Pt 1), 1533-1536. doi:10.1016/j.juro.2007.05.116

Kuo, H. C., Liu, H. T., & Chancellor, M. B. (2010). Urinary nerve growth factor is a better biomarker than detrusor wall thickness for the assessment of overactive bladder with incontinence. *Neurourol Urodyn,* **29**(3), 482-487. doi:10.1002/nau.20741

Lee, H., Koh, B. H., Peri, L. E., Sanders, K. M., & Koh, S. D. (2014). Purinergic inhibitory regulation of murine detrusor muscles mediated by PDGFRalpha+ interstitial cells. *J Physiol,* **592**(Pt 6), 1283-1293. doi:10.1113/jphysiol.2013.267989

Lee, H. Y., Bardini, M., & Burnstock, G. (2000). Distribution of P2X receptors in the urinary bladder and the ureter of the rat. *J Urol,* **163**(6), 2002-2007.

Levesque, S. A., Lavoie, E. G., Lecka, J., Bigonnesse, F., & Sevigny, J. (2007). Specificity of the ecto-ATPase inhibitor ARL 67156 on human and mouse ectonucleotidases. *Br J Pharmacol,* **152**(1), 141-150. doi:10.1038/sj.bjp.0707361

Levin, R. M., Ruggieri, M. R., & Wein, A. J. (1986). Functional effects of the purinergic innervation of the rabbit urinary bladder. *J Pharmacol Exp Ther,* **236**(2), 452-457.

Lewis, S. A., & Lewis, J. R. (2006). Kinetics of urothelial ATP release. *Am J Physiol Renal Physiol,* **291**(2), F332-340. doi:00340.2005 [pii] 10.1152/ajprenal.00340.2005

Li, Y., Xue, L., Miao, Q., Mao, F., Yao, L., Yuan, J., Qin, W., Zhao, Y., Sun, H., Liu, F., Wang, H. (2013). Expression and electrophysiological characteristics of P2X3 receptors in interstitial cells of Cajal in rats with partial bladder outlet obstruction. *BJU Int,* **111(**5), 843-851. doi:10.1111/j.1464-410X.2012.11408.x

Liu, H. T., Chancellor, M. B., & Kuo, H. C. (2009). Urinary nerve growth factor levels are elevated in patients with detrusor overactivity and decreased in responders to detrusor botulinum toxin-A injection. *Eur Urol,* **56**(4), 700-706. doi:S0302-2838(08)00484-3 [pii] 10.1016/j.eururo.2008.04.037

Liu, H. T., Chen, C. Y., & Kuo, H. C. (2010). Urinary nerve growth factor levels in overactive bladder syndrome and lower urinary tract disorders. *J Formos Med Assoc,* **109**(12), 862-878. doi:S0929-6646(10)60133-7 [pii] 10.1016/S0929-6646(10)60133-7

Lloyd, H. G., & Fredholm, B. B. (1995). Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochem Int,* **26**(4), 387-395. doi:019701869400144J [pii]

Loffler, M., Morote-Garcia, J. C., Eltzschig, S. A., Coe, I. R., & Eltzschig, H. K. (2007). Physiological roles of vascular nucleoside transporters.

Arterioscler Thromb Vasc Biol, **27**(5), 1004-1013. doi:ATVBAHA.106.126714 [pii] 10.1161/ATVBAHA.106.126714

Lu, S. H., Groat, W. C., Lin, A. T., Chen, K. K., & Chang, L. S. (2007). Evaluation of purinergic mechanism for the treatment of voiding dysfunction: a study in conscious spinal cord-injured rats. *J Chin Med Assoc,* **70**(10), 439-444. doi:S1726-4901(08)70035-2 [pii]

Lukacsko, P., & Krell, R. D. (1982). Response of the guinea-pig urinary bladder to purine and pyrimidine nucleotides. *Eur J Pharmacol,* **80**(4), 401-406.

Magalhães-Cardoso, M. T., Pereira, M. F., Oliveira, L., Ribeiro, J. A., Cunha, R. A., & Correia-de-Sá, P. (2003). Ecto-AMP deaminase blunts the ATP-derived adenosine A2A receptor facilitation of acetylcholine release at rat motor nerve endings. *J Physiol,* **549**(Pt 2), 399-408. doi:10.1113/jphysiol.2003.040410 2003.040410 [pii]

Mansfield, K. J., Liu, L., Mitchelson, F. J., Moore, K. H., Millard, R. J., & Burcher, E. (2005). Muscarinic receptor subtypes in human bladder detrusor and mucosa, studied by radioligand binding and quantitative competitive RT-PCR: changes in ageing. *Br J Pharmacol,* **144**(8), 1089-1099. doi:10.1038/sj.bjp.0706147

Masuda H, Chancellor MB, Kihara K, de Groat WC, & N, Y. (2005). Evidence for the involvement of spinal endogenous ATP and P2X receptors in detrusor overactivity caused by acetic acid, acrolein or cyclophosphamide. *Neurourol Urodyn,* **24**(5-6). doi:10.1002/nau.20173

McMurray, G., Dass, N., & Brading, A. F. (1998). Purinoceptor subtypes mediating contraction and relaxation of marmoset urinary bladder smooth muscle. *Br J Pharmacol,* **123**(8), 1579-1586. doi:10.1038/sj.bjp.0701774

Meng, M., Zheng, J., Yan, J., Li, Q., Fang, Q., & Li, W. (2015). P2X and P2X Receptors Mediate Bladder Hyperesthesia in ICC in Female Overactive Bladder. *Cell Biochem Biophys*, **72**(2),375-83. doi:10.1007/s12013-014-0471-x

Michel, M. C., & Barendrecht, M. M. (2008). Physiological and pathological regulation of the autonomic control of urinary bladder contractility. *Pharmacol Ther,* **117**(3), 297-312. doi:10.1016/j.pharmthera.2007.12.001

Mirone, V., Imbimbo, C., Longo, N., & Fusco, F. (2007). The detrusor muscle: an innocent victim of bladder outlet obstruction. *Eur Urol,* **51**(1), 57-66. doi:S0302-2838(06)00890-6 [pii] 10.1016/j.eururo.2006.07.050

Mirone, V., Imbimbo, C., Sessa, G., Palmieri, A., Longo, N., Granata, A. M., & Fusco, F. (2004). Correlation between detrusor collagen content and urinary symptoms in patients with prostatic obstruction. *J Urol,* **172**(4 Pt 1), 1386-1389.

Mohlin, C., Save, S., Nilsson, M., & Persson, K. (2009). Studies of the extracellular ATP-adenosine pathway in human urinary tract epithelial cells. *Pharmacology,* **84**(4), 196-202. doi:000235908 [pii] 10.1159/000235908

Moldwin R, Kitt M, Mangel J, Beyer R, Hanno P, Butera P, & A, F. (2015). A phase 2 study in women with interstitial cystitis/bladder pain syndrome (IC/PBS) of the novel P2X3 antagonist AF-219. *Neurourol Urodyn,* **34**(S3), S50.

Montecucco, C., & Molgo, J. (2005). Botulinal neurotoxins: revival of an old killer. *Curr Opin Pharmacol,* **5**(3), 274-279. doi:10.1016/j.coph.2004.12.006

Moore, C. K., Levendusky, M., & Longhurst, P. A. (2002). Relationship of mass of obstructed rat bladders and responsiveness to adrenergic stimulation. *J Urol,* **168**(4 Pt 1), 1621-1625. doi:10.1097/01.ju.0000029753.21156.45

Moore, K. H., Gilpin, S. A., Dixon, J. S., Richmond, D. H., & Sutherst, J. R. (1992). Increase in presumptive sensory nerves of the urinary bladder in idiopathic detrusor instability. *Br J Urol,* **70**(4), 370-372.

Moore, K. H., Richmond, D. H., & Sutherst, J. (1990). Urinary acidity in relation to detrusor instability. *Neurourol Urodyn,* **9**(4), 331-332. doi:10.1002/nau.1930090402

Mori, A., & Shindou, T. (2003). Modulation of GABAergic transmission in the striatopallidal system by adenosine A2A receptors: a potential mechanism for the antiparkinsonian effects of A2A antagonists. *Neurology,* **61**(11 Suppl 6), S44-48.

Munoz, A., Gangitano, D. A., Smith, C. P., Boone, T. B., & Somogyi, G. T. (2010). Removal of urothelium affects bladder contractility and release of ATP but not release of NO in rat urinary bladder. *BMC Urol,* **10**, 10. doi:1471-2490-10-10 [pii] 10.1186/1471-2490-10-10

Munoz, A., Somogyi, G. T., Boone, T. B., Ford, A. P., & Smith, C. P. (2012). Modulation of bladder afferent signals in normal and spinal cord-injured rats by purinergic P2X3 and P2X2/3 receptors. *BJU Int,* **110**(8 Pt B), E409-414. doi:10.1111/j.1464-410X.2012.11189.x

Murakami, S., Yoshida, M., Masunaga, K., Maeda, Y., & Ueda, S. (2008). Change in acetylcholine release from rat bladder with partial outlet obstruction. *BJU Int,* **101**(5), 633-639. doi:BJU7325 [pii] 10.1111/j.1464-410X.2007.07325.x

Nakamura, F., & Strittmatter, S. M. (1996). P2Y1 purinergic receptors in sensory neurons: contribution to touch-induced impulse generation. *Proc Natl Acad Sci U S A,* **93**(19), 10465-10470.

Namasivayam, S., Eardley, I., & Morrison, J. F. (1999). Purinergic sensory neurotransmission in the urinary bladder: an in vitro study in the rat. *BJU Int,* **84**(7), 854-860.

Neuhaus, J., Schulte-Baukloh, H., Stolzenburg, J. U., Speroni di Fenizio, P., Horn, L. C., Ruffert, H., Hartenstein, S., Burger, M., Schulze, M., Schwalenberg, T. (2012). Individual receptor profiling as a novel tool to support diagnosis of bladder pain syndrome/interstitial cystitis (BPS/IC). *World J Urol,* **30**(5), 693-700. doi:10.1007/s00345-011-0774-0

Nicholls, J., Hourani, S. M., & Kitchen, I. (1992). Characterization of P1-purinoceptors on rat duodenum and urinary bladder. *Br J Pharmacol,* **105**(3), 639-642.

Nitti, V. W., Dmochowski, R., Herschorn, S., Sand, P., Thompson, C., Nardo, C., Yan, X., Haag-Molkenteller, C., Embark Study Group (2013). OnabotulinumtoxinA for the treatment of patients with overactive bladder and urinary incontinence: results of a phase 3, randomized, placebo controlled trial. *J Urol,* **189**(6), 2186-2193. doi:10.1016/j.juro.2012.12.022

Nitti, V. W., Rovner, E. S., & Bavendam, T. (2010). Response to fesoterodine in patients with an overactive bladder and urgency urinary incontinence is independent of the urodynamic finding of detrusor overactivity. *BJU Int,* **105**(9), 1268-1275. doi:BJU9037 [pii] 10.1111/j.1464-410X.2009.09037.x

O'Reilly, B. A., Kosaka, A. H., Chang, T. K., Ford, A. P., Popert, R., & McMahon, S. B. (2001). A quantitative analysis of purinoceptor expression in the

bladders of patients with symptomatic outlet obstruction. *BJU Int,* **87**(7), 617-622. doi:bju179 [pii]

Ochodnicky, P., Cruz, C. D., Yoshimura, N., & Michel, M. C. (2011). Nerve growth factor in bladder dysfunction: contributing factor, biomarker, and therapeutic target. *Neurourol Urodyn,* **30**(7), 1227-1241. doi:10.1002/nau.21022

Ockrim, J. L., Laniado, M. E., Patel, A., Tubaro, A., & St Clair Carter, S. (2001). A probability based system for combining simple office parameters as a predictor of bladder outflow obstruction. *J Urol,* **166**(6), 2221-2225.

Oelke, M. (2010). International Consultation on Incontinence-Research Society (ICI-RS) report on non-invasive urodynamics: the need of standardization of ultrasound bladder and detrusor wall thickness measurements to quantify bladder wall hypertrophy. *Neurourol Urodyn,* **29**(4), 634-639. doi:10.1002/nau.20834

Oelke, M., Hofner, K., Jonas, U., de la Rosette, J. J., Ubbink, D. T., & Wijkstra, H. (2007). Diagnostic accuracy of noninvasive tests to evaluate bladder outlet obstruction in men: detrusor wall thickness, uroflowmetry, postvoid residual urine, and prostate volume. *Eur Urol,* **52**(3), 827-834. doi:10.1016/j.eururo.2006.12.023

Osman, N. I., Chapple, C. R., Abrams, P., Dmochowski, R., Haab, F., Nitti, V., Koelbl, H., van Kerrebroeck, P., Wein, A. J. (2014). Detrusor underactivity and the underactive bladder: a new clinical entity? A review of current terminology, definitions, epidemiology, aetiology, and diagnosis. *Eur Urol,* **65**(2), 389-398. doi:10.1016/j.eururo.2013.10.015

Owen, S. J., Massa, H. H., & Rose'Meyer, R. B. (2012). Loss of adenosine A2B receptor mediated relaxant responses in the aged female rat bladder; effects of dietary phytoestrogens. *Naunyn Schmiedebergs Arch Pharmacol,* **385**(5), 539-549. doi:10.1007/s00210-011-0722-y

Palea, S., Corsi, M., Pietra, C., Artibani, W., Calpista, A., Gaviraghi, G., & Trist, D. G. (1994). ADP beta S induces contraction of the human isolated urinary bladder through a purinoceptor subtype different from P2X and P2Y. *J Pharmacol Exp Ther,* **269**(1), 193-197.

Pandita, R. K., & Andersson, K. E. (2002). Intravesical adenosine triphosphate stimulates the micturition reflex in awake, freely moving rats. *J Urol,* **168**(3), 1230-1234. doi:10.1097/01.ju.0000023398.35234.ea S0022-5347(05)64631-9 [pii]

Pannek, J., Janek, S., Sommerer, F., & Tannapfel, A. (2009). Expression of purinergic P2X2-receptors in neurogenic bladder dysfunction due to spinal cord injury: a preliminary immunohistochemical study. *Spinal Cord,* **47**(7), 561-564. doi:10.1038/sc.2008.165

Parija, S. C., Raviprakash, V., & Mishra, S. K. (1991). Adenosine- and alpha,beta-methylene ATP-induced differential inhibition of cholinergic and non-cholinergic neurogenic responses in rat urinary bladder. *Br J Pharmacol,* **102**(2), 396-400.

Patel, A. K., Patterson, J. M., & Chapple, C. R. (2006). Botulinum toxin injections for neurogenic and idiopathic detrusor overactivity: A critical analysis of results. *Eur Urol,* **50**(4), 684-709; discussion 709-610. doi:10.1016/j.eururo.2006.07.022

Peng, W., Cotrina, M. L., Han, X., Yu, H., Bekar, L., Blum, L., Takano, T., Tian, G. F., Goldman, S. A., Nedergaard, M. (2009). Systemic administration of

an antagonist of the ATP-sensitive receptor P2X7 improves recovery after spinal cord injury. *Proc Natl Acad Sci U S A,* **106**(30), 12489-12493. doi:10.1073/pnas.0902531106

Peters, T. J., Donovan, J. L., Kay, H. E., Abrams, P., de la Rosette, J. J., Porru, D., & Thuroff, J. W. (1997). The International Continence Society "Benign Prostatic Hyperplasia" Study: the botherosomeness of urinary symptoms. *J Urol,* **157**(3), 885-889.

Phelps, P. T., Anthes, J. C., & Correll, C. C. (2006). Characterization of adenosine receptors in the human bladder carcinoma T24 cell line. *Eur J Pharmacol,* **536**(1-2), 28-37. doi:S0014-2999(06)00172-5 [pii] 10.1016/j.ejphar.2006.02.046

Pinheiro, A. R., Paramos-de-Carvalho, D., Certal, M., Costa, M. A., Costa, C., Magalhães-Cardoso, M. T., Ferreirinha, F., Sevigny, J., Correia-de-Sá, P. (2013). Histamine induces ATP release from human subcutaneous fibroblasts, via pannexin-1 hemichannels, leading to Ca2+ mobilization and cell proliferation. *J Biol Chem,* **288**(38), 27571-27583. doi:M113.460865 [pii] 10.1074/jbc.M113.460865

Pinto, R., Lopes, T., Frias, B., Silva, A., Silva, J. A., Silva, C. M., Cruz, C., Cruz, F., Dinis, P. (2010). Trigonal injection of botulinum toxin A in patients with refractory bladder pain syndrome/interstitial cystitis. *Eur Urol,* **58**(3), 360-365. doi:10.1016/j.eururo.2010.02.031

Prakasam, H. S., Herrington, H., Roppolo, J. R., Jackson, E. K., & Apodaca, G. (2012). Modulation of bladder function by luminal adenosine turnover and A1 receptor activation. *Am J Physiol Renal Physiol,* **303**(2), F279-292. doi:ajprenal.00566.2011 [pii] 10.1152/ajprenal.00566.2011

Ralevic, V., & Burnstock, G. (1998). Receptors for purines and pyrimidines. *Pharmacol Rev,* **50**(3), 413-492.

Reitz, A., Stohrer, M., Kramer, G., Del Popolo, G., Chartier-Kastler, E., Pannek, J., . . . Schurch, B. (2004). European experience of 200 cases treated with botulinum-A toxin injections into the detrusor muscle for urinary incontinence due to neurogenic detrusor overactivity. *Eur Urol,* **45**(4), 510-515. doi:10.1016/j.eururo.2003.12.004

Ribeiro, J. A., Cunha, R. A., Correia-de-Sá, P., & Sebastião, A. M. (1996). Purinergic regulation of acetylcholine release. *Prog Brain Res,* **109**, 231-241.

Robson, S. C., Sevigny, J., & Zimmermann, H. (2006). The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic Signal,* **2**(2), 409-430. doi:10.1007/s11302-006-9003-5

Rocha, I., Burnstock, G., & Spyer, K. M. (2001). Effect on urinary bladder function and arterial blood pressure of the activation of putative purine receptors in brainstem areas. *Auton Neurosci,* **88**(1-2), 6-15. doi:S1566-0702(00)00284-8 [pii] 10.1016/S1566-0702(00)00284-8

Roosen, A., Datta, S. N., Chowdhury, R. A., Patel, P. M., Kalsi, V., Elneil, S., Dasgupta, P., Kessler, T. M., Khan, S., Panicker, J., Fry, C. H. Brandner, S., Fowler, C. J., Apostolidis, A. (2009). Suburothelial myofibroblasts in the human overactive bladder and the effect of botulinum neurotoxin type A treatment. *Eur Urol,* **55**(6), 1440-1448. doi:S0302-2838(08)01314-6 [pii] 10.1016/j.eururo.2008.11.009

Rosen, R., Altwein, J., Boyle, P., Kirby, R. S., Lukacs, B., Meuleman, E., O'Leary,

M. P., Puppo, P., Robertson, C., Giuliano, F. (2003). Lower urinary tract symptoms and male sexual dysfunction: the multinational survey of the aging male (MSAM-7). *Eur Urol,* **44**(6), 637-649.

Rosier, P. F., & de la Rosette, J. J. (1995). Is there a correlation between prostate size and bladder-outlet obstruction? *World J Urol,* **13**(1), 9-13.

Rovner, E., Kennelly, M., Schulte-Baukloh, H., Zhou, J., Haag-Molkenteller, C., & Dasgupta, P. (2011). Urodynamic results and clinical outcomes with intradetrusor injections of onabotulinumtoxinA in a randomized, placebo-controlled dose-finding study in idiopathic overactive bladder. *Neurourol Urodyn,* **30**(4), 556-562. doi:10.1002/nau.21021

Rubinstein, R., Shalev, M., Nissenkorn, I., & Cohen, S. (1998). Effect of exogenous adenosine and its monophosphate on the contractile response to acetylcholine in the human isolated detrusor muscle strips. *J Auton Pharmacol,* **18**(2), 99-104.

Ruggieri, M. R., Sr. (2006). Mechanisms of disease: role of purinergic signaling in the pathophysiology of bladder dysfunction. *Nat Clin Pract Urol,* **3**(4), 206-215. doi:10.1038/ncpuro0456

Ruggieri, M. R., Sr., & Braverman, A. S. (2006). Regulation of bladder muscarinic receptor subtypes by experimental pathologies. *Auton Autacoid Pharmacol,* **26**(3), 311-325. doi:AAP377 [pii] 10.1111/j.1474-8673.2006.00377.x

Sadananda, P., Shang, F., Liu, L., Mansfield, K. J., & Burcher, E. (2009). Release of ATP from rat urinary bladder mucosa: role of acid, vanilloids and stretch. *Br J Pharmacol,* **158**(7), 1655-1662. doi:BPH431 [pii] 10.1111/j.1476-5381.2009.00431.x

Saito, M., Yokoi, K., Ohmura, M., & Kondo, A. (1997). Effects of partial outflow obstruction on bladder contractility and blood flow to the detrusor: comparison between mild and severe obstruction. *Urol Int,* **59**(4), 226-230.

Save, S., Mjosberg, J., Poljakovic, M., Mohlin, C., & Persson, K. (2009). Adenosine receptor expression in Escherichia coli-infected and cytokine-stimulated human urinary tract epithelial cells. *BJU Int,* **104**(11), 1758-1765. doi:BJU8638 [pii] 10.1111/j.1464-410X.2009.08638.x

Schulte, G., & Fredholm, B. B. (2003). Signalling from adenosine receptors to mitogen-activated protein kinases. *Cell Signal,* **15**(9), 813-827. doi:S0898656803000585 [pii]

Sears, C. L., Lewis, C., Noel, K., Albright, T. S., & Fischer, J. R. (2010). Overactive bladder medication adherence when medication is free to patients. *J Urol,* **183**(3), 1077-1081. doi:10.1016/j.juro.2009.11.026

Shabir, S., Cross, W., Kirkwood, L. A., Pearson, J. F., Appleby, P. A., Walker, D., Eardley, I., Southgate, J. (2013). Functional expression of purinergic P2 receptors and transient receptor potential channels by the human urothelium. *Am J Physiol Renal Physiol,* **305**(3), F396-406. doi:10.1152/ajprenal.00127.2013

Sheth, S., Brito, R., Mukherjea, D., Rybak, L. P., & Ramkumar, V. (2014). Adenosine receptors: expression, function and regulation. *Int J Mol Sci,* **15**(2), 2024-2052. doi:10.3390/ijms15022024

Silva, I., Correia, J., Ferreirinha, F., Magalhães-Cardoso, M. T., Silva-Ramos, M., Sévigny, J., & Correia-de-Sá, P. (2011). Bladder overactivity due to global impairment of ecto-NTPDases in humans with lower urinary tract disorders

exhibiting unbalanced ATP/adenosine formation. *Auton Neurosci Basic Clinical,* **163**, 49-50.

Silva, I., Ferreirinha, F., Magalhães-Cardoso, M. T., Silva-Ramos, M., & Correia-de-Sá, P. (2015). Activation of P2Y6 Receptors Facilitates Nonneuronal Adenosine Triphosphate and Acetylcholine Release from Urothelium with the Lamina Propria of Men with Bladder Outlet Obstruction. *J Urol,* **194**(4), 1146-1154. doi:10.1016/j.juro.2015.05.080

Silva-Ramos, M., Cavadas, V., & Abranches-Monteiro, L. (2013a). Clinical and urodynamic variables associated with female bladder outlet obstruction. *Neurourol Urodyn,* **32**(6), 756. doi:10.1002/nau.22472

Silva-Ramos, M., Silva, I., Duarte-Araújo, M., Magalhães-Cardoso, T., Lafuente-de-Carvalho, J., & Correia-de-Sá, P. (2010). Loss of balance between excitatory P2X3 and inhibitory A1 receptors contribute to cholinergic hyperactivity in obstructed human bladder. *Basic Clin Pharmacol Toxicol,* **107** (supplement s1), Abstract nº2397.

Silva-Ramos, M., Silva, I., Faria, M., Magalhães-Cardoso, M. T., Correia, J., Ferreirinha, F., & Correia-de-Sá, P. (2015a). Impairment of ATP hydrolysis decreases adenosine A1 receptor tonus favoring cholinergic nerve hyperactivity in the obstructed human urinary bladder. *Purinergic Signal,* **11**(4), 595-606. doi:10.1007/s11302-015-9478-z

Silva-Ramos, M., Silva, I., Faria, M., Magalhães-Cardoso, M. T., & Correia-de-Sá, P. (2015b). Urinary bladder disorders: Is adenosine friend or foe? In K. Warrick (Ed.), *Adenosine receptors: Pharmacology, functions and therapeutic aspects* (1st ed., pp. 115-142). Hauppauge, NY: Nova Science Publishers Inc.

Silva-Ramos, M., Silva, I., Ferreirinha, F., Faria, M., & Correia-de-Sá, P. (2016a). Overactive bladder (OAB): Is there a place for drugs targeting the purinergic cascade? In L. E. (Ed.), *Overactive Bladder (OAB): Prevalence, Risk Factors and Management* (1st ed.). Hauppauge, NY: Nova Science Publishers Inc.

Silva-Ramos, M., Silva, I., Oliveira, J. C., & Correia-de-Sá, P. (2016b). Increased urinary adenosine triphosphate in patients with bladder outlet obstruction due to benign prostate hyperplasia. *Prostate*, **76**(15), 1353-63. doi:10.1002/pros.23207

Silva-Ramos, M., Silva, I., Oliveira, O., Ferreira, S., Reis, M. J., Oliveira, J. C., & Correia-de-Sá, P. (2013b). Urinary ATP may be a dynamic biomarker of detrusor overactivity in women with overactive bladder syndrome. *PLoS One,* **8**(5), e64696. doi:10.1371/journal.pone.0064696

Simpson, L. L. (1979). The action of botulinal toxin. *Rev Infect Dis,* **1**(4), 656-662.

Siroky, M. B., Olsson, C. A., & Krane, R. J. (1979). The flow rate nomogram: I. Development. *J Urol,* **122(**5), 665-668.

Sitkovsky, M. V., Lukashev, D., Apasov, S., Kojima, H., Koshiba, M., Caldwell, C., Ohta, A., Thiel, M. (2004). Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. *Annu Rev Immunol,* **22**, 657-682. doi:10.1146/annurev.immunol.22.012703.104731

Sjogren, C., Andersson, K. E., Husted, S., Mattiasson, A., & Moller-Madsen, B. (1982). Atropine resistance of transmurally stimulated isolated human bladder muscle. *J Urol,* **128**(6), 1368-1371.

Smet, P. J., Moore, K. H., & Jonavicius, J. (1997). Distribution and colocalization of calcitonin gene-related peptide, tachykinins, and vasoactive intestinal peptide in normal and idiopathic unstable human urinary bladder. *Lab Invest,* **77**(1), 37-49.

Smith, C. P., Franks, M. E., McNeil, B. K., Ghosh, R., de Groat, W. C., Chancellor, M. B., & Somogyi, G. T. (2003). Effect of botulinum toxin A on the autonomic nervous system of the rat lower urinary tract. *J Urol,* **169**(5), 1896-1900. doi:10.1097/01.ju.0000049202.56189.54 S0022-5347(05)63710-X [pii]

Smith, C. P., Vemulakonda, V. M., Kiss, S., Boone, T. B., & Somogyi, G. T. (2005). Enhanced ATP release from rat bladder urothelium during chronic bladder inflammation: effect of botulinum toxin A. *Neurochem Int,* **47**(4), 291-297. doi:10.1016/j.neuint.2005.04.021

Smith, D. O., & Lu, Z. (1991). Adenosine derived from hydrolysis of presynaptically released ATP inhibits neuromuscular transmission in the rat. *Neurosci Lett,* **122**(2), 171-173.

Smith, S. A., Black, D., Song, D., and Ford, A. P. (2013). *A novel method to estimate AF-219 levels in bladder interstitium derived from drug excreted into the urine.* FASEB J. **27**, 1103.

Sosnowski, M., & Yaksh, T. L. (1990). The role of spinal and brainstem adenosine receptors in the modulation of the volume-evoked micturition reflex in the unanesthetized rat. *Brain Res,* **515**(1-2), 207-213. doi:0006-8993(90)90597-5 [pii]

Sperlágh, B., Heinrich, A., & Csolle, C. (2007). P2 receptor-mediated modulation of neurotransmitter release-an update. *Purinergic Signal,* **3**(4), 269-284. doi:10.1007/s11302-007-9080-0

Sperlágh, B., & Vizi, E. S. (1991). Effect of presynaptic P2 receptor stimulation on transmitter release. *J Neurochem,* **56**(5), 1466-1470.

Steers, W. D., Kolbeck, S., Creedon, D., & Tuttle, J. B. (1991). Nerve growth factor in the urinary bladder of the adult regulates neuronal form and function. *J Clin Invest,* **88**(5), 1709-1715. doi:10.1172/JCI115488

Stefan, C., Jansen, S., & Bollen, M. (2005). NPP-type ectophosphodiesterases: unity in diversity. *Trends Biochem Sci,* **30**(10), 542-550. doi:S0968-0004(05)00240-9 [pii] 10.1016/j.tibs.2005.08.005

Stehle, J. H., Rivkees, S. A., Lee, J. J., Weaver, D. R., Deeds, J. D., & Reppert, S. M. (1992). Molecular cloning and expression of the cDNA for a novel A2-adenosine receptor subtype. *Mol Endocrinol,* **6**(3), 384-393.

Stewart, W. F., Van Rooyen, J. B., Cundiff, G. W., Abrams, P., Herzog, A. R., Corey, R., Hunt, T. L., Wein, A. J. (2003). Prevalence and burden of overactive bladder in the United States. *World J Urol,* **20**(6), 327-336. doi:10.1007/s00345-002-0301-4

Sugaya, K., Nishijima, S., Kadekawa, K., Miyazato, M., & Mukouyama, H. (2009). Relationship between lower urinary tract symptoms and urinary ATP in patients with benign prostatic hyperplasia or overactive bladder. *Biomed Res,* **30**(5), 287-294. doi:JST.JSTAGE/biomedres/30.287 [pii]

Sugaya, K., Nishijima, S., Tasaki, S., Kadekawa, K., Miyazato, M., & Ogawa, Y. (2007). Effects of propiverine and naftopidil on the urinary ATP level and bladder activity after bladder stimulation in rats. *Neurosci Lett,* **429**(2-3), 142-146. doi:S0304-3940(07)01096-8 [pii] 10.1016/j.neulet.2007.10.003

Sui, G., Fry, C. H., Montgomery, B., Roberts, M., Wu, R., & Wu, C. (2014). Purinergic and muscarinic modulation of ATP release from the urothelium and its paracrine actions. *Am J Physiol Renal Physiol,* **306**(3), F286-298. doi:10.1152/ajprenal.00291.2013

Sui, G. P., Wu, C., & Fry, C. H. (2006). Characterization of the purinergic receptor subtype on guinea-pig suburothelial myofibroblasts. *BJU Int,* **97**(6), 1327-1331. doi:BJU6200 [pii] 10.1111/j.1464-410X.2006.06200.x

Sultana, R. V., Zalstein, S., Cameron, P., & Campbell, D. (2001). Dipstick urinalysis and the accuracy of the clinical diagnosis of urinary tract infection. *J Emerg Med,* **20**(1), 13-19.

Sun, Y., Keay, S., De Deyne, P. G., & Chai, T. C. (2001). Augmented stretch activated adenosine triphosphate release from bladder uroepithelial cells in patients with interstitial cystitis. *J Urol,* **166**(5), 1951-1956. doi:S0022-5347(05)65726-6 [pii]

Sun, Y., MaLossi, J., Jacobs, S. C., & Chai, T. C. (2002). Effect of doxazosin on stretch-activated adenosine triphosphate release in bladder urothelial cells from patients with benign prostatic hyperplasia. *Urology,* **60**(2), 351-356. doi:S0090429502017107 [pii]

Suzuki, H., & Kokubun, S. (1994). Subtypes of purinoceptors in rat and dog urinary bladder smooth muscles. *Br J Pharmacol,* **112**(1), 117-122.

Thomas, A. W., Cannon, A., Bartlett, E., Ellis-Jones, J., & Abrams, P. (2004). The natural history of lower urinary tract dysfunction in men: the influence of detrusor underactivity on the outcome after transurethral resection of the prostate with a minimum 10-year urodynamic follow-up. *BJU Int,* **93**(6), 745-750. doi:10.1111/j.1464-410X.2003.04719.x

Thompson, I. M., Ankerst, D. P., Chi, C., Lucia, M. S., Goodman, P. J., Crowley, J. J., Parnes, H. L., Coltman, C. A., Jr. (2005). Operating characteristics of prostate-specific antigen in men with an initial PSA level of 3.0 ng/ml or lower. *JAMA,* **294**(1), 66-70. doi:10.1001/jama.294.1.66

Thore, A., Ansehn, S., Lundin, A., & Bergman, S. (1975). Detection of bacteriuria by luciferase assay of adenosine triphosphate. *J Clin Microbiol,* **1**(1), 1-8.

Timóteo, M. A., Carneiro, I., Silva, I., Noronha-Matos, J. B., Ferreirinha, F., Silva-Ramos, M., & Correia-de-Sá, P. (2014). ATP released via pannexin-1 hemichannels mediates bladder overactivity triggered by urothelial P2Y6 receptors. *Biochem Pharmacol,* **87**(2), 371-379. doi:10.1016/j.bcp.2013.11.007

Truschel, S. T., Wang, E., Ruiz, W. G., Leung, S. M., Rojas, R., Lavelle, J., Zeidel, M., Stoffer, D., Apodaca, G. (2002). Stretch-regulated exocytosis/endocytosis in bladder umbrella cells. *Mol Biol Cell,* **13**(3), 830-846. doi:10.1091/mbc.01-09-0435

Turner, W. H., & Brading, A. F. (1997). Smooth muscle of the bladder in the normal and the diseased state: pathophysiology, diagnosis and treatment. *Pharmacol Ther,* **75**(2), 77-110.

Uvin, P., Boudes, M., Menigoz, A., Franken, J., Pinto, S., Gevaert, T., Verplaetse, R., Tytgat, J., Vennekens, R., Voets, T., De Ridder, D. (2013). Chronic administration of anticholinergics in rats induces a shift from muscarinic to purinergic transmission in the bladder wall. *Eur Urol,* **64**(3), 502-510. doi:S0302-2838(13)00497-1 [pii] 10.1016/j.eururo.2013.05.031

van Koeveringe, G. A., Vahabi, B., Andersson, K. E., Kirschner-Herrmans, R., & Oelke, M. (2011). Detrusor underactivity: a plea for new approaches to a

common bladder dysfunction. *Neurourol Urodyn,* **30**(5), 723-728. doi:10.1002/nau.21097

Van Mastrigt, R., & Rollema, H. J. (1992). The prognostic value of bladder contractility in transurethral resection of the prostate. *J Urol,* **148**(6), 1856-1860.

Vesela, R., Aronsson, P., & Tobin, G. (2011). Functional and morphological examinations of P1A1 purinoceptors in the normal and inflamed urinary bladder of the rat. *Auton Neurosci,* **159**(1-2), 26-31. doi:S1566-0702(10)00139-6 [pii] 10.1016/j.autneu.2010.07.008

Vial, C., & Evans, R. J. (2000). P2X receptor expression in mouse urinary bladder and the requirement of P2X(1) receptors for functional P2X receptor responses in the mouse urinary bladder smooth muscle. *Br J Pharmacol,* **131**(7), 1489-1495. doi:10.1038/sj.bjp.0703720

Vidotto, C., Fousert, D., Akkermann, M., Griesmacher, A., & Muller, M. M. (2003). Purine and pyrimidine metabolites in children's urine. *Clin Chim Acta,* **335**(1-2), 27-32. doi:S0009898103002912 [pii]

Vieira, C., Magalhães-Cardoso, M. T., Ferreirinha, F., Silva, I., Dias, A. S., Pelletier, J., Sevigny, J., Correia-de-Sá, P. (2014). Feed-forward inhibition of CD73 and upregulation of adenosine deaminase contribute to the loss of adenosine neuromodulation in postinflammatory ileitis. *Mediators Inflamm,* **2014**, 254640. doi:10.1155/2014/254640

Vlaskovska, M., Kasakov, L., Rong, W., Bodin, P., Bardini, M., Cockayne, D. A., Ford, A.P., Burnstock, G. (2001). P2X3 knock-out mice reveal a major sensory role for urothelially released ATP. *J Neurosci,* **21**(15), 5670-5677. doi:21/15/5670 [pii]

Wang, E., Truschel, S., & Apodaca, G. (2003). Analysis of hydrostatic pressure-induced changes in umbrella cell surface area. *Methods,* **30**(3), 207-217. doi:S1046202303000276 [pii]

Wang, E. C., Lee, J. M., Ruiz, W. G., Balestreire, E. M., von Bodungen, M., Barrick, S., Cockayne, D.A., Birder, L.A., Apodaca, G. (2005). ATP and purinergic receptor-dependent membrane traffic in bladder umbrella cells. *J Clin Invest,* **115**(9), 2412-2422. doi:10.1172/JCI24086

Wedenberg, K., Ronquist, G., Waldenstrom, A., & Ulmsten, U. (1994). Low energy charge and high adenosine content in smooth muscle of human bladder in comparison with striated muscle. *Int J Clin Lab Res,* **24**(4), 230-232.

Williams, M. (1987). Purine receptors in mammalian tissues: pharmacology and functional significance. *Annu Rev Pharmacol Toxicol,* **27**, 315-345. doi:10.1146/annurev.pa.27.040187.001531

Wiseman, O. J., Fowler, C. J., & Landon, D. N. (2003). The role of the human bladder lamina propria myofibroblast. *BJU Int,* **91**(1), 89-93. doi:3802 [pii]

Wu, C., Bayliss, M., Newgreen, D., Mundy, A. R., & Fry, C. H. (1999). A comparison of the mode of action of ATP and carbachol on isolated human detrusor smooth muscle. *J Urol,* **162**(5), 1840-1847. doi:S0022-5347(05)68248-1 [pii]

Wu, C., Gui, G. P., & Fry, C. H. (2011). Intracellular Ca(2+) regulation and electrophysiolgical properties of bladder urothelium subjected to stretch and exogenous agonists. *Cell Calcium,* **49**(6), 395-399. doi:S0143-4160(11)00057-1 [pii] 10.1016/j.ceca.2011.03.008

Wust, M., Averbeck, B., Reif, S., Brater, M., & Ravens, U. (2002). Different responses to drugs against overactive bladder in detrusor muscle of pig, guinea pig and mouse. *Eur J Pharmacol,* **454**(1), 59-69. doi:S0014299902024780 [pii]

Yamaguchi, O., Nomiya, M., & Andersson, K. E. (2014). Functional consequences of chronic bladder ischemia. *Neurourol Urodyn,* **33**(1), 54-58. doi:10.1002/nau.22517

Yang, S. J., An, J. Y., Shim, J. O., Park, C. H., Huh, I. H., & Sohn, U. D. (2000). The mechanism of contraction by 2-chloroadenosine in cat detrusor muscle cells. *J Urol,* **163**(2), 652-658. doi:S0022-5347(05)67952-9 [pii]

Yokota, T., & Yamaguchi, O. (1996). Changes in cholinergic and purinergic neurotransmission in pathologic bladder of chronic spinal rabbit. *J Urol,* **156**(5), 1862-1866. doi:S0022-5347(01)65554-X [pii]

Yoshida, M., Homma, Y., Inadome, A., Yono, M., Seshita, H., Miyamoto, Y., . . . Ueda, S. (2001). Age-related changes in cholinergic and purinergic neurotransmission in human isolated bladder smooth muscles. *Exp Gerontol,* **36**(1), 99-109.

Yoshida, M., Miyamae, K., Iwashita, H., Otani, M., & Inadome, A. (2004). Management of detrusor dysfunction in the elderly: changes in acetylcholine and adenosine triphosphate release during aging. *Urology,* **63** (3 Suppl 1), 17-23. doi:10.1016/j.urology.2003.11.003 S0090429503012172 [pii]

Youden, W. J. (1950). Index for rating diagnostic tests. *Cancer,* **3**(1), 32-35.

Yu, W., Robson, S. C., & Hill, W. G. (2011). Expression and distribution of ectonucleotidases in mouse urinary bladder. *PLoS One,* **6**(4), e18704. doi:10.1371/journal.pone.0018704

Yu, W., Sun, X., Robson, S. C., & Hill, W. G. (2013). Extracellular UDP enhances P2X-mediated bladder smooth muscle contractility via P2Y(6) activation of the phospholipase C/inositol trisphosphate pathway. *FASEB J,* **27**(5), 1895-1903. doi:10.1096/fj.12-219006

Yu, W., Zacharia, L. C., Jackson, E. K., & Apodaca, G. (2006). Adenosine receptor expression and function in bladder uroepithelium. *Am J Physiol Cell Physiol,* **291**(2), C254-265. doi:00025.2006 [pii] 10.1152/ajpcell.00025.2006

Yu, Y. B., Zuo, X. L., Zhao, Q. J., Chen, F. X., Yang, J., Dong, Y. Y., Wang, P., Li, Y. Q. (2012). Brain-derived neurotrophic factor contributes to abdominal pain in irritable bowel syndrome. *Gut,* **61**(5), 685-694. doi:10.1136/gutjnl-2011-300265

Zhang, Q., Siroky, M., Yang, J. H., Zhao, Z., & Azadzoi, K. (2014). Effects of ischemia and oxidative stress on bladder purinoceptors expression. *Urology,* **84**(5), 1249 e1241-1247. doi:10.1016/j.urology.2014.07.023

Zhang, S. X., Kobayashi, T., Okada, T., Garcia del Saz, E., & Seguchi, H. (1991). Alkaline phosphatase, 5'-nucleotidase and magnesium-dependent adenosine triphosphatase activities in the transitional epithelium of the rat urinary bladder. *Histol Histopathol,* **6**(3), 309-315.

Zhang, Y., & Xia, Y. (2012). Adenosine signaling in normal and sickle erythrocytes and beyond. *Microbes Infect,* **14**(10), 863-873. doi:S1286-4579(12)00123-2 [pii] 10.1016/j.micinf.2012.05.005

Zhou, Y., Murthy, J. N., Zeng, D., Belardinelli, L., & Blackburn, M. R. (2010). Alterations in adenosine metabolism and signaling in patients with chronic

obstructive pulmonary disease and idiopathic pulmonary fibrosis. *PLoS One,* **5**(2), e9224. doi:10.1371/journal.pone.0009224

Zimmermann, H. (2000). Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol,* **362**(4-5), 299-309.